



**João Pedro Pereira Mendes**

Bachelor of Science in Chemical and Biochemical Engineering

## **Chromatographic purification of virus particles for advanced therapy medicinal products**

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Adviser: Ricardo Silva, Post-Doc Researcher, ITQB-UNL

Co-adviser: Cristina Peixoto, Competence Driven R&D Lab Head,  
ITQB-UNL

Chairperson: Professor Mário Fernando José Eusébio, FCT-UNL

Opponent: Professor José Paulo Barbosa Mota, FCT-UNL



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CIÊNCIAS E TECNOLOGIA  
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To my father



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## Abstract

The increasing number of cancer diagnoses in the last decades is associated with behavioral risks, in addition with those genetically originated. The fight against the disease usually relapses in not selective mechanisms like chemotherapy or radiotherapy, that induce the organism in deep alterations and side effects. One of the alternative strategies relies on the use of advanced therapies medicinal products suchlike viruses, to carry out the treatment in each cell taking advantage of their invading abilities. The cost of producing this oncolytic virus directly influences process engineering, and thus, numerous efforts have been made to improve each purification step.

The development of the downstream process begins with the clarification of the viruses harvested from the bioreactor with two depth-filtration steps. This allows a gradual removal of larger impurities like cell debris with a complete virus recovery. Afterwards, a tangential flow filtration step enables volume reduction. After concentration, the retentate is subjected to diafiltration which allows not only the permeation of impurities but also the formulation of the concentrated product for the next processing step.

The following step in the purification train is anion exchange chromatography. The chromatographic media used was selected after successive screening tests with a library of resins and membranes. The conditions used reflect the study carried out, in the sense that the load employed corresponds to the DBC<sup>10%</sup> obtained of  $6.2 \times 10^{11}$  (TP/ml) particles per millilitre and the elution of the viruses is preceded by a low salt concentration elution (200 mM) in order to remove impurities. The yield obtained is 85%. The purification process ends with polishing and sterile filtration to achieve the specified conditions, through a size-exclusion chromatography and membrane filters, respectively, obtaining a total yield of 53%.

The study also opens perspectives on innovation and future development with the performance of multi-column chromatography assays and automated filtration tests, both in specialized equipment.



## Resumo

O aumento do número de diagnósticos de cancro nas últimas décadas deve-se a riscos comportamentais, em associação aos originados geneticamente. O combate à doença recai comumente em mecanismos pouco seletivos como quimioterapia ou radioterapia, que induzem no organismo profundas alterações e efeitos secundários. Uma das estratégias alternativas recai na utilização de vírus modificados para realizar o tratamento em cada célula, tirando partido das capacidades invasoras dos vírus. O custo de produção destes vírus oncolíticos influencia diretamente a engenharia de processo, e assim sendo, inúmeros esforços têm sido enveredados no sentido de melhorar e otimizar cada passo da purificação.

O desenvolvimento do processo de downstream inicia-se com a clarificação dos vírus recolhidos do bioreator por meio de dois *depth-filters*. Este passo permite a remoção gradual de impurezas maiores com uma completa recuperação de vírus. Seguidamente, um conjunto otimizado de filtrações tangenciais permite redução do volume. Após a concentração, o retentado é submetido a diafiltrações que possibilitam não apenas a permeação de impurezas, mas também a formulação do produto concentrado para o próximo passo de processamento.

O passo seguinte consiste na purificação cromatográfica por troca iónica. O meio cromatográfico utilizado foi selecionado após testes sucessivos com uma biblioteca de resinas e membranas. As condições utilizadas refletem o estudo efetuado no sentido em que carga empregue corresponde à  $DBC^{10\%}$  obtida de  $6.2 \times 10^{11}$  TP/ml (partículas por mililitro) e a eluição dos vírus propriamente ditos é precedida de uma eluição com pouca concentração de sal (200 mM) de forma a retirar grande parte das impurezas. O rendimento obtido é de 85%. O processo de purificação termina com polimento e filtração estéril tendo como objetivo alcançar as condições especificadas, através de cromatografia de exclusão molecular e filtros de membrana, respetivamente, tendo sido obtido um rendimento total de 53%.

O estudo efetuado abre ainda perspetivas sobre inovação e desenvolvimento futuro com a realização de ensaios de cromatografia multi-coluna e filtração automatizada, ambos em equipamentos especializados.



## Motivation

The uprising number of diagnosed patients and cancer-related deaths appeals for increased dedication and research on this field; besides the clear necessity for better treatments, scientific interest and clinical development have never been so pronounced.

R&D for oncolytic virus therapies appeared at the beginning of the 20<sup>th</sup> century, “surrounded” by theoretical potential and hope for an alternative strategy for the emerging cancer diseases. One hundred years later, the development is remarkable, with some already being clinically available. But a lot has yet to be done. Despite all efforts, and mainly due to huge production and purification costs, the therapy is too expensive, and therefore, hardly affordable.

By focusing on improving and optimizing downstream processes, researchers gradually reduce its costs, increase overall economic viability, and try to make therapies more accessible to patients and healthcare systems.



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## List of Abbreviations

<b>AEX</b>	Anion-exchange Chromatography
<b>BSA</b>	Bovine Serum Albumin
<b>CIP</b>	Cleaning-in-place
<b>CV</b>	Column Volume
<b>DNA</b>	Deoxyribonucleic Acid
<b>FDA</b>	Food and Drug Association
<b>GBM</b>	Glioblastomas
<b>GF</b>	Gel Filtration
<b>GMP</b>	Good Manufacturing Practices
<b>GS</b>	Gliosarcoma
<b>HIC</b>	Hydrophobicity Interaction Chromatography
<b>HPLC</b>	High-Performance Liquid Chromatography
<b>IEX</b>	Ion-exchange Chromatography
<b>IZAC</b>	Immobilized Zinc Affinity chromatography
<b>MAMC</b>	Metal Affinity Membrane Chromatography
<b>MCSCGP</b>	Multicolumn Solvent Gradient Chromatography
<b>NTA</b>	Nanoparticles Tracking Analysis
<b>PBS</b>	Phosphate Buffered Saline
<b>QC</b>	Quality Control
<b>q-PCR</b>	Real Time PCR
<b>R&amp;D</b>	Research and Development
<b>SEC</b>	Size-exclusion chromatography
<b>SMB</b>	Simulated Moving Bed
<b>TFF</b>	Tangential Flow Filtration
<b>TMP</b>	Transmembrane Pressure



# 1

# Introduction

*This chapter contextualizes the research on its scientific environment through bioprocess analyses and a literature review on the latest purification development for oncolytic viruses, complemented with an outline in modelling and multicolumn chromatography.*





## 1.1 Cancer and Oncolytic Therapies

Cancer is one of the most notorious and fast growing diseases enhanced by the dietary and behavioural risks of nowadays society like high body mass index, lack of physical activity or tobacco and alcohol use [1,2]. Presenting itself as the second biggest death cause in 2015, with more than 8.8 million registered cases, cancer is responsible for almost 1 in every 6 deaths [1].

Traditional and more common treatments include chemo and radiation therapies, which through medication or intense radiation doses try to kill cancer cells or reduce tumours [3]. Although some satisfactory results can be achieved, their pathway is dangerous and may induce other systemic problems due to the amount and severity of dosage or lack of specificity for cancer/tumour cells. So new treatments have been developed, such as hormone therapy or stem cells transplant [3]. Among these innovative strategies are oncolytic virus therapies which take advantage of pathogenic agents to induce specific behaviours on target cells. By genetically engineering the perfect modified virus, scientists can promote virus replication and cell lysis dependent on the infected cell being normal or cancerous [4]. This is one of the most important advantages of virus therapy as it reduces toxicity and effects on non-cancerous cells [5,6].

Virus particles have been growing as a platform for drug production and thoroughly investigated as one of the most promising approaches for gene therapy vectors and viral vaccines [7]. The amount of possibilities brought by the multiple oncolytic viruses available, like reovirus or adenovirus, gives researchers a good amount of ways to solve tumours' killing problems, from oncolysis amplification to gene therapy (Fig. 1).

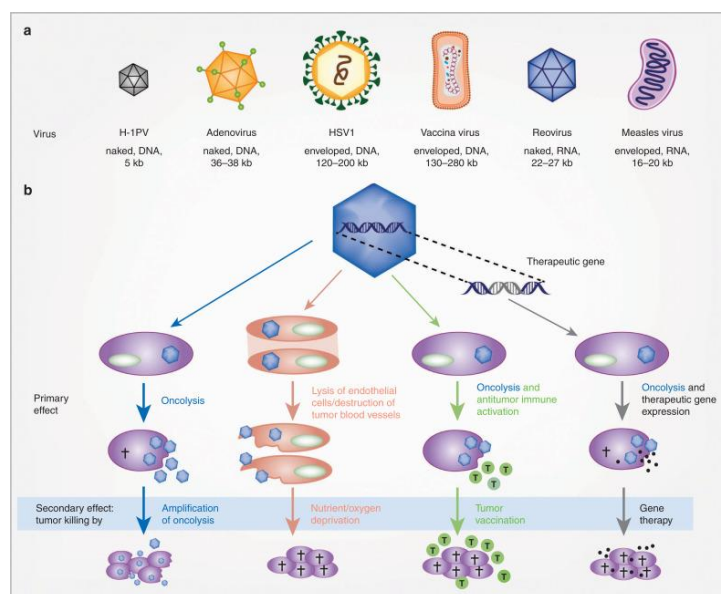


Figure 1 - Oncolytic viruses diversity (a) and implemented modes of action (b) [8]

Targeting mechanisms and tumour selectivity are driven by several factors depending on the chosen oncolytic virus. Researchers' first concern is the cell entry mechanism, being via virus-specific or receptor-mediated. Tumour cells often express viral receptors, and while the increased selectivity for those highly decreases immune responses, it also increases success possibility. Other factors include rapid cell division inside tumour cells or deficiencies in antiviral type I. Even though these might seem problematic, the increased cell division may induce higher virus replication selectivity in tumour cells. Associated with the lack of enough antiviral, it can, upon cell lysis, release virus among cell debris, developing immunosuppression near the tumour and inducing pathways for antitumor immunity [9].

As research and interest grow on this topic, so does the range of studied viruses as potential candidates for cancer therapeutics. Examples of these are Vaccinia, Herpes or Adenovirus, with many published clinical trials, and other ones like Polio and Parvovirus, still without any reported [9].

The first therapy approved by the FDA (the United States Food and Drug Administration) that uses oncolytic viruses is Imlygic (talimogene laherparepvec). This "first-of-its-kind" therapy targets metastatic melanoma using modified live oncolytic herpes virus on lesions that couldn't be completely removed by surgery [10]. The main advantage of this approach is the limited toxicity associated with the "ability to use each individual tumour as a source of antigen to generate a highly specific antitumor immune response" [11].

The interest on these new treatments is reflected on researchers' publications and clinical trials ongoing. Regarding adenovirus vectors, the amount of clinical trials increased from approximately 25% in the early 2000's [12,13], to a 50% growth per year since 2012, on gene therapy targeting cancer [14]. Reported studies presented results on tumour/cancer cells located on bone (osteosarcoma) [15], brain [16], skin (melanoma) [17] and head or neck [18]. In fact, world's first adenovirus oncolytic therapy was approved in China, back in 2005, to treat head or neck cancer. This treatment paired oncolytic virus with chemotherapy, increasing response rates to almost 80% compared to 40% when prescribing only the standard procedure. The oncolytic used was H101, an adenovirus serotype 5 with deleted E3 gene [19,20].

Studied adenovirus differ on the modification made and targeted cells. Strategies contrast from deletion or insertion to modification of specific genes and having many variable goals like nonmuscle invasive bladder cancer, diffuse intrinsic pontine glioma or recurrent malignant gynaecologic diseases. Nowadays, a substantial number of clinical trials using some

of these adenoviruses are taking place. Currently, in phase 1, trials conducted by VCN Biosciences, S.L. and DNAtix, Inc., are measuring primarily safety/tolerability and efficacy, on therapies developed to target specifically advanced solid tumours and recurrent brain, glioblastomas (GBM) or gliosarcoma (GS) [9].

Recombinant adenoviruses are gaining relevance as gene transfer vectors for gene therapy owing to its characteristics for this purpose [21]. Morphologically being non-enveloped with a diameter from 70 to 120 nanometres, and presenting double-stranded DNA associated with high transductions efficiencies and gene expression levels [12,22,23], makes this kind of virus particles great candidates for innovative virotherapies [7].

## 1.2 Upstream Process

The production of oncolytic adenovirus can be achieved by many producer cells [24,25], such as PER.C6, C139 or the most known HEK293 which allows a high titter of productivity [8]. Another commonly used cell line is A549 which has been documented for GMP manufacturing and was used to produce the oncolytic virus studied in the scope of this thesis. These producer cells have been reported in several publications regarding adenovirus for oncolytic therapies (Table 1).

Table 1 - Adenovirus production cells [26]

PRODUCER CELL	ORIGIN	REFERENCE
<b>HEK293</b>	Human embryonic kidney with sheared adenovirus DNA	Russell et al., 1977 [27]
<b>PER.C6</b>	Human embryonic retinoblast cells with a shorter adenoviral sequence	Fallaux et al., 1998 [28]
<b>A549</b>	Human lung cancer cells	Imler et al., 2005 [29]
<b>C139</b>	Derived from A549	Farson et al., 2005 [30]

Their production methods have been thoroughly reported with each one being particularly better in some parameters or for specific cell lines. Depending on the working scale, the approaches may include spinners, roller bottles and bioreactors [8].

### 1.3 Downstream Process

The exponential awareness for oncolytic virus therapies catalyses the interest on developing scalable purification processes capable of ensuring compliance with product's demand for clinical grade scale [31]. Although many different strategies are reported in the literature, the most usual purification train uses five key process stages such as the ones depicted in Figure 2 [8].

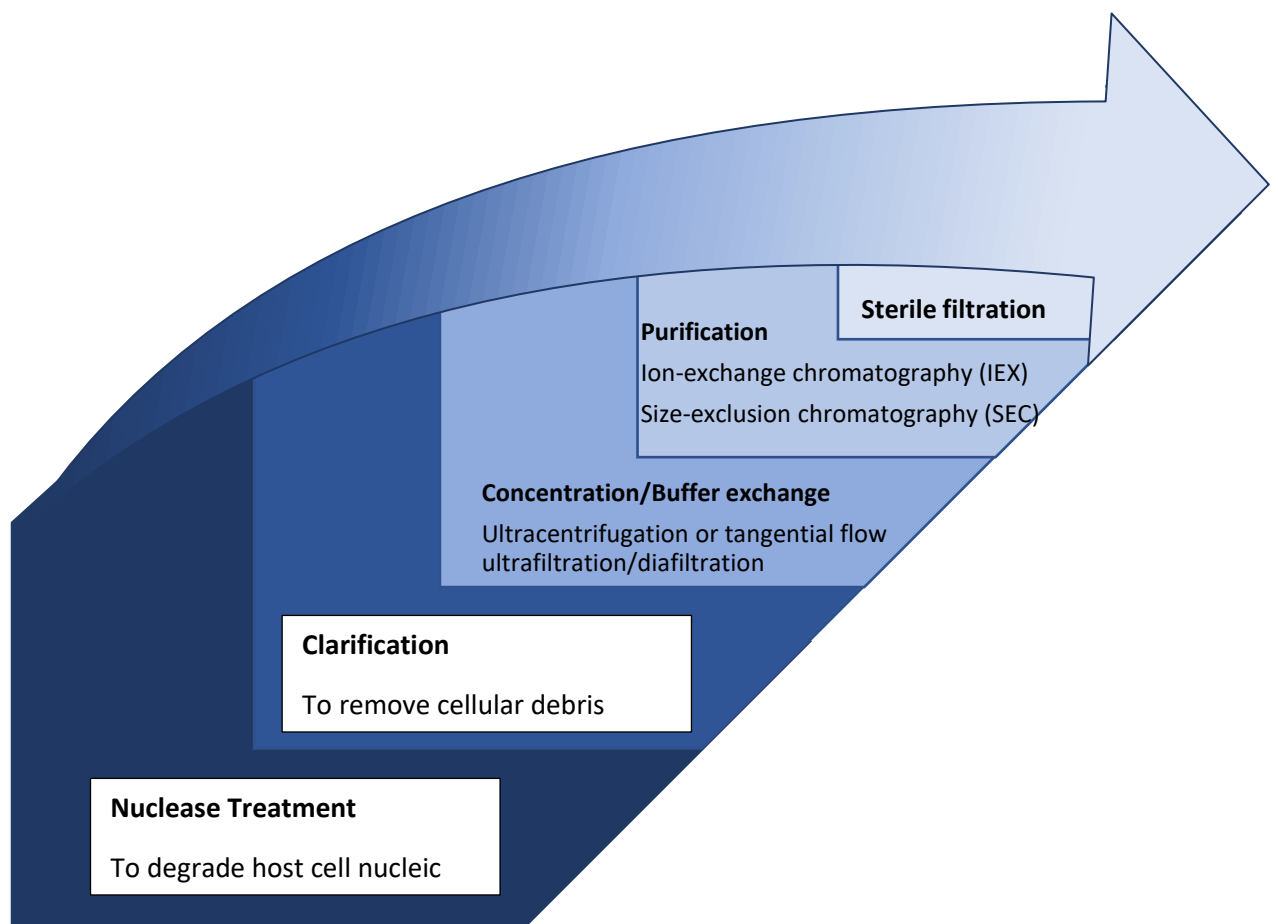


Figure 2 - Common five steps of oncolytic viruses purification process [13]

Although most of the emphasis of the downstream process is put in the intermediate purification stages - chromatography, the improvement of the lysis, nuclease treatment, clarification or concentration steps have shown great repercussion on overall processes performance.

The first step after bioreaction is cell lysis. Several strategies can be found in the literature to perform this operation. The most common are the use of freeze/thaw cycles followed by water baths [12,13,32,33]. Surfactants such as Triton X-100 [34] which can be directly applied on the bioreactor or coupled with centrifugation and/or a brief sonication after the freeze/thaw cycles [35] have also been recognized for their potential as cell lysis agents.

Nuclease treatment is particularly important for adenovirus production, since host cell's DNA may theoretically increase potential tumorigenicity [36]. Although other methods can be considered, for example, anion-exchange procedures, the most common are nuclease treatments with Benzonase (EM Science) and DNA precipitation. Firstly, through the usage of this endonuclease, both DNA and RNA are digested which assists in achieving FDA guidelines for nucleic acid contamination. Secondly, it can be introduced in many different solutions due to stability and activeness in a large range of conditions [37], making it reliable for adenovirus purification either in research or clinical trials[36]. However, it has been reported some difficulty in separating benzonase remains from cell lysates [38]. Selective DNA precipitation using ammonium sulphate and polyethylene glycol was also demonstrated [39] with good separation yields achieved [40].

The objective of clarification stage is to simultaneously remove cell debris or large products (e.g. process-related aggregates) in an efficient way and to ensure product quality in the flowthrough [7]. The two most common processes are centrifugation and membrane filtration. As scalability is a major deciding factor in research and development studies, both strategies have been tried on different scales [41]. These multiple options are been considered since the 90's and different approaches have been tested from centrifugation [32] to more versatile and robust depth filters [34].

Concentration allows reduction of clarified bulk by successful removing low-molecular-weight process' impurities like host-cell proteins or fragmented cell DNA. Using pre-selected filters and filtration conditions (pore size, TMP and shear-rate), it's possible to meaningfully reduce the volume while increasing volumetric concentration of virus particles [41]. Volume reduction decreases upfront investments on downstream processes costs [7] while increasing significantly overall viability. Associated with filtration processes, it's common to introduce a

step of buffer exchange, which allows the formulation of the virus bulk for the next processing steps.

## 1.4 Intermediate purification

Virus intermediate purification processes have been improved in every aspect since early 1960's when were mainly based on density gradients [42]. These processes included two rounds of cesium chloride (CsCl) or sucrose density gradient ultracentrifugation following freeze-thaw cycles to disrupt infected cells [43,44]. Among several process' disadvantages, such as poor yields, inconstant degrees of purity [33] and the necessity of consequent CsCl removal [45], the main concern was related to the lack of scale up viability [46,47]. Nevertheless, density gradient ultracentrifugation is still performed for research and pre-clinical applications. Moreover, small chromatography kits are available for the same scale enabling improvements on purified volume and total yield while reducing total operation time in more than 90%, to only 3 hours [48].

New strategies have been tried to overcome this problem, some with more success than others. Unsuccessful approaches include filtration processes, which were developed from the principle that viruses are comparatively larger particles than impurities present. These strategies failed on technical aspects, namely because filters were designed with the purpose of virus removal. Additionally, particle aggregation on the membrane surface greatly contributes to viral loss [49].

A successful way to purify adenovirus, providing great process adaptability and scale up viability is through chromatographic processes. These include column and high-performance liquid chromatography (HPLC) both allowing purification through several different characteristics, such as size-exclusion (SEC) [50], ion exchange (IEX) [51], affinity [12], hydrophobic interaction (HIC) [32], or processes combining more than one of them [13,31]. Reported reviews have already been made to compare different strategies [32,49] (Table 2).

Table 2 - Chromatography Strategies' Comparison [32]

<b>Virus Type</b>	<b>ACN53</b>			
<b>Cell Line</b>	<b>ATCC 293</b>			
<b>Purification Method</b>	<b>Resin</b>	<b>Buffers</b>	<b>Elution</b>	<b>Yield</b>
<b>SEC</b>	Toyopearl HW-75F	PBS (2% Sucrose, 2 mM MgCl <sub>2</sub> )	n.a.	15 – 20 %
<b>AEX</b>	Fractogel DEAE-650 M	50 mM HEPES ( <b>pH 7.5</b> , 300 mM NaCl, 2 mM MgCl <sub>2</sub> , 2% sucrose) 50 mM HEPES ( <b>pH 8</b> , 300 mM NaCl, 2 mM MgCl <sub>2</sub> , 2% sucrose)	Gradient	n.a.
<b>HIC</b>	Toyopearl butyl 650M  Toyopearl phenyl 650M	50 mM Tris (pH 8, 3 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> )	Gradient	5 – 30 %
<b>Affinity</b>	IZAC column with zinc/glycine system	50 mM HEPES (pH 7.5, <b>450 mM NaCl</b> , 2 mM MgCl <sub>2</sub> , 2% sucrose) 50 mM HEPES (pH 7.5, <b>150 mM NaCl</b> , 2 mM MgCl <sub>2</sub> , 2% sucrose)	Gradient	47%

Chromatography columns allow for products discrimination through a large variety of characteristics supported on the required difference between itself and remaining components (impurities).

Size-exclusion procedures are considered for a wide variety of ends depending on which stage of the process are applied; at the beginning, in clarification or ultrafiltration, at this

stage in purification by chromatography, or later on polishing and sterile filtration. Size-exclusion, as the name suggests, allows for fractionation of different molecules or compounds depending on their dimensions and individualization of the product from other components, like DNA or proteins. The initial solution is injected through a precise arrangement of materials, that have a defined pore, and in some cases, intra-pore size.

Regarding resins, as an example, their packed bed has accountable volume, and through research and experimentation, it's possible to arrive at precise measurements of dead volume, pore size, intra-pore size and others, which then, will truly define the size-exclusion specifications required for a purification process. The following figure (Fig. 3) presents the outcome of a SEC column, being the first peak the pretended product and the remain, impurities. The first image represents the results of eluted volume according to the molecular weight of present biomolecules. With this analysis, it's verifiable that the viruses go through the matrix while impurities and other calibration molecules are caught on it which justifies their delay.

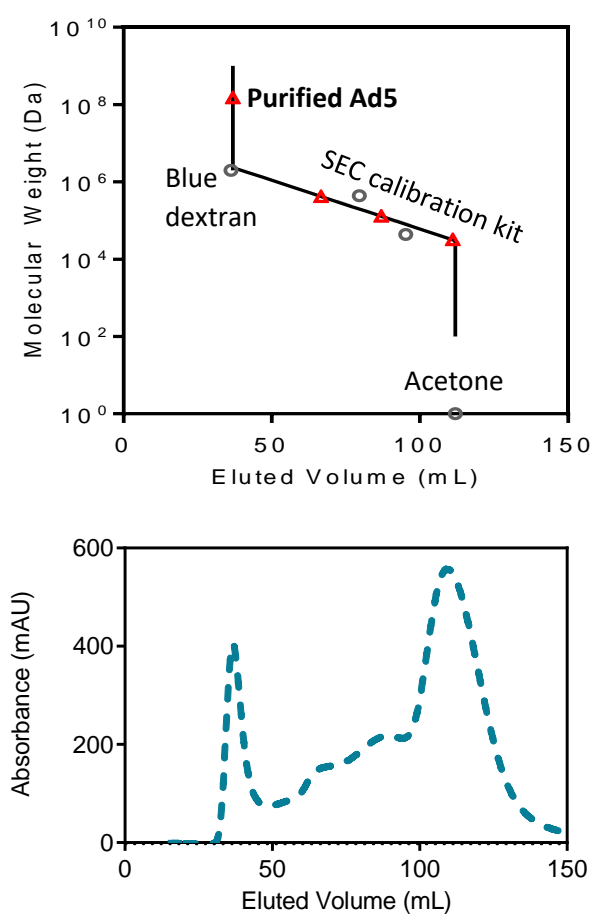


Figure 3 – HPLC-SEC chromatography profile (wavelength 280 nm) [7]



Purification strategies based on membrane or surface charge, allow for product separation through a stationary phase that adsorbs opposite charged particles. By previously studying particle's charge, using, for instance, Zeta-potential assays, the right conditions can be achieved and product's purity can be maximized. Depending on the column being positively or negatively charged, IEX chromatography is narrowly named anion-exchange (AEX) or cation-exchange (CEX), respectively. A great number of research has been developed using AEX chromatography for adenovirus purification, with presented results showing improvements regarding purity, virus particles' and infection units' yields [33,34,51,53,54].

Differently from size-exclusion, adsorption based processes require a second phase called desorption. This procedure allows product's retrieval through the introduction of a perturbation, either being related to salt concentration, pH or any other. The induce change will unbalance the equilibriums in-place and as result release the adsorbed particles.

Hydrophobicity interaction chromatography (HIC) relies on "reversible interaction between a protein and the hydrophobic ligand" [53]. This strategy has been studied as intermediary purification step [32], as its conditions are quite mild compared to others in order to preserve biological activity, and offer the advantage of being optimized by higher salt concentration as the ones encountered after elution on a previous purification column [53].

Affinity chromatography is used complementarily to other strategies and is often associated with capture and polishing steps. Regarding adenovirus, metal affinity strategies have been tried against regular purification processes consisting in CsCl density gradients with great results [12]. This purification strategy depends on specific interactions between targeted molecule and column's ligand. In this particular case, this bond is established between virus capsid and specific biological ligand coupled to the chromatographic matrix [49]. Like IEX and HIC, at the end of the adsorption step, the column is saturated and a clear majority of impurities have already been washed. Then through buffering change, the elution step results in a product with great purity through high selectiveness.

Chromatography strategies depend on the right conditions to purify the intended compound. The solutions used are adjusted through a variety of parameters such as salt concentration, pH and main species present, which usually names the buffer. The most reliable for biochemical systems have been described, and its choice is supported by some main characteristics concerning with pKa, solubility or effects with other components [54]. Reported studies have shown an immense variety of solutions for each step of a chromatographic procedure, constituted mainly by solutions of HEPES (4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid) or TRIS (2-Amino-2-(hydroxymethyl)-1,3-propanediol). Different buffers allow good stability in a considerable operation range; for instance regarding pH, HEPES sustains equilibrium between 6.8 and 8.2 and TRIS from 7 to 9 [55].

Ionic strength is a major factor on buffer's constitution and one of the biggest differentiation aspects between chromatography strategies. Regarding AEX targeting adenovirus purification, equilibration and washing steps have been reported with a variety of NaCl concentrations. Taking that into account, to ensure better process' flexibility, research studies describe different introductions of salt concentrations through a gradient to the maximum of 400 mM of sodium chloride [7,12,13,23]. Concerning elution, different methods can be applied to elute adsorbed virus particles. Considering that the process is being conducted at a specific percentage of reagent B, if it's raised gradually within a period, until reaching an established concentration, the elution was made with a gradient, otherwise, if B's concentration is increased immediately, it's called a step elution. Usually, strategies make use of gradient elution from washing/equilibration concentration until around 600 mM [23,32,41].

Besides salt and main component, buffers' composition often includes few other substances which have specific contributions to the overall process. Most of these are related to adenovirus stability and include sucrose,  $\text{MgCl}_2$  and glycerol. Sucrose, for instance, has been mentioned within a correlation study between concentration and "thermally induced structural changes", arriving at the conclusion that increasing sucrose concentration stabilizes secondary and tertiary structures despite the fact that at 45 degrees Celsius, the quaternary structure is lost [56]. Regarding  $\text{MgCl}_2$ , early studies included it on buffer's formulations as a stabilizer [57]. However, more recent approaches to the matter couldn't conclude association between both [58,59]. Nevertheless, 1 mM of  $\text{MgCl}_2$  was reported as virus capsid stabilizer under high-temperature stress conditions [36,58]. Concerning glycerol, previous research has shown that it enhances virus integrity through capsid stabilization and so is often used in final formulation [13,60,61].

## 1.5 Overview of different purification pathways

Considering previous explanation about the major key steps on a purification strategy, it becomes relevant to enhance different approaches and adaptations, that could introduce improvements and present themselves as future possibilities on adenovirus purification. Its production starts within the bioreactor's bulk and until product's final formulation is achieved many different pathways can be taken to the generally used process presented below (Fig. 4).

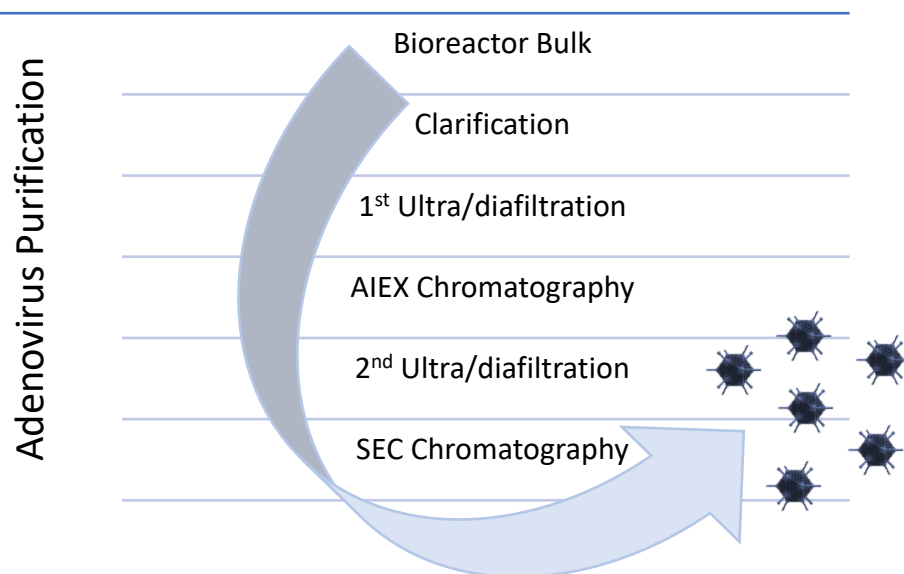


Figure 4 - Purification process for adenovirus using chromatography [7]

Using the purification process depicted in Figure 4 as a starting point, substitutions or suppressions can be made with the purpose of improving overall purification, reducing operation time, optimizing parameters, complying with the final formulation, or anything else that increments all around robustness and viability. Different approaches to previous steps have already been addressed, becoming now relevant to focus on chromatography and polishing.

Regarding substitutions to the purification train, a great number of studies have been reported since *Huyghe et al.* [32] described chromatographic purification strategies using five different resins, from the conventional AEC (Source 15 Q) to more complex approaches like Immobilized Zinc Affinity chromatography (IZAC) or HIC [32]. Since then, changes regarding different chromatographic medias, process parameters and buffers allowed process viability and potential scale ability to use in clinical trials.

As an example, *Eglon et al.* [13] compared the previous strategies with the non-scalable method of caesium chloride density gradient centrifugation [13,48] while searching for the optimum order between AEX and size exclusion chromatography (SEC). Even though CsCl purification results in higher vector yield, it shows less recuperation after desalting, a lower ratio between total virion and infectious particles and less viability for scale-up. Focusing on chromatography strategies, and starting with the conventional order (AEX-SEC), the results were comparable to the obtained with gradient ultracentrifugation but contrasted with a reverse association (SEC-AEX) which obtained less purity and required further treatment [13].

*Lee et al.* [12] approached polishing strategies. The reported work describes an improved procedure supported on metal affinity membrane chromatography (MAMC) as a replacement for the more common, and perhaps “established”, SEC chromatography [12], making an overview of the entire chromatography spectrum focusing on yields and purity. As the main difference, membrane chromatography presents larger pore sizes and the possibility to operate at higher flow rates, while remaining the support for relevant ligands. The study directly compared membrane and resin affinity chromatography resulting in a 32% increased yield on membrane usage. Associated with this improvement, it's theorized that the usage of MAMC as a rapid flow-through process to remove empty capsids (defective particles) can be a source of viability, affecting clinical consistency on adenovirus provisions [12,62].

As mentioned before, besides improvement through regular process optimization, reported studies showed focus on suppression of different steps, introducing new platforms or techniques that allow multiple levels of purification in only one or, for instance, a counter-current operation of the chromatographic system. Examples of this strategies were demonstrated by *Peixoto et al.* [23] and *Nestola et al.* [7], presenting results about expanded bed chromatography and simulated countercurrent chromatography using two SEC columns, respectively.

Expanded bed chromatography introduces an alternative to early purification steps, allowing suppression of clarification and concentration associating them to the initial chromatography [23]. Reports of virus lost from 25 to 80 percent, during harvest due to early cell lysis [63], enhances needed optimization of such strategies. This can be done directly from the unclarified medium, as it's reported. This strategy consists of a stable fluidized bed that allows particles from the feedstock to pass through, introducing several degrees of purification, permitting for a single step to combine the usual two or three. Associated with reduction of steps, are increased economic viability, overall process yields and reduction of expended time, all supporting expanded bed chromatography as a viable and attractive technique for cost-effective processes [23,64].

### 1.5.1 Multicolumn chromatography

Another approach was taken by *Nestola et al.* using countercurrent chromatography and simulated moving bed (SMB) technology, to create a quasi-continuous process with two SEC columns for adenovirus purification. SMB, as a chromatography concept for purification, has

been reported for a great variety of purposes, from the production of plasmid DNA (pDNA) [65] to influenza virus [66], with more general and amplified studies also being made considering it as a possible strategy for the future of bioseparation [67,68]. SMB allows higher productivity and yields through maximization of column's used capacity while reducing both buffer's consumption and needed column's volume [7]. These advances reduce overall investment and increase attractiveness, justifying more research on this approach as a purification strategy for bioprocesses [66,69,70].

In a countercurrent chromatography system, while the mobile phase flows in opposite direction of the stationary phase, the solutes will be selectively attracted to one or another phase. The relation between phases and components is figuratively explained by Figure 5. As it's demonstrated in single column elution chromatography, the outcoming product may lack enough differentiation from remaining impurities. On the other side, countercurrent chromatography establishes different phase "motions" and allows for better separation results, as shown both in column's chromatography profile (Fig. 5 b, c).

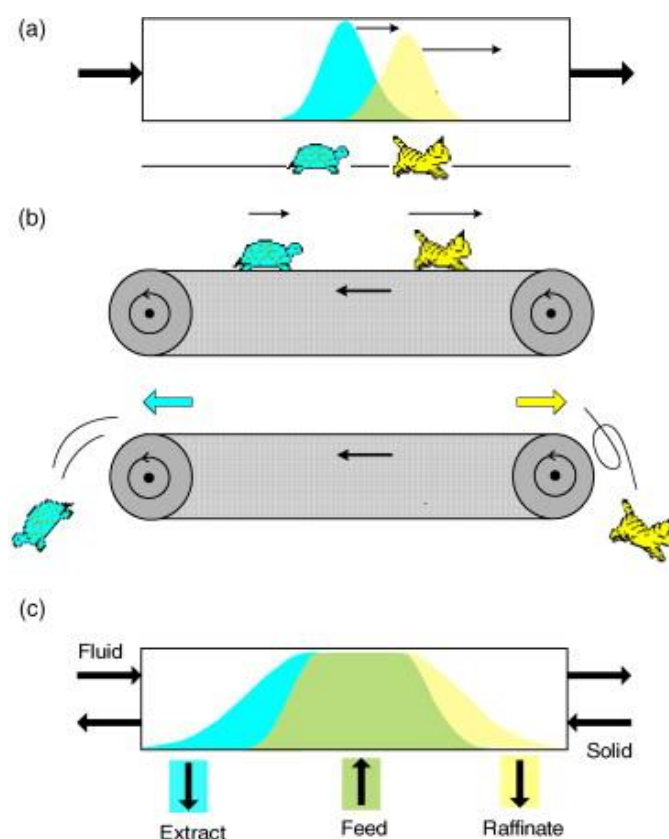


Figure 5 - Elution (a) and Countercurrent Chromatography (b, c) [71]

Taking advantage of this scenario, a cycle of columns can be arranged to ensure selective separation for a specific set of conditions, introducing complexity and optimization while allowing better yields and productivity, as mentioned before.

The two-column, in open-loop, SEC purification step by *Nestola et al.*, included 10 two-way valves which controlled intended flows, 2 HPLC pumps (one for sample and one for fresh mobile phase), 2 UV detectors on the effluent of each column and 2 SEC's columns, as shown in Figure 6. This scheme narrows column's flow through to one of three situations; either it is frozen, directed to the other column, or being diverted to the product or the waste lines [7]. When compared to a single batch SEC, the reported approach led to similar results in what regards impurity removal but allowed for a 51% increase in virus yield with a 6-fold increment to column's productivity [7].

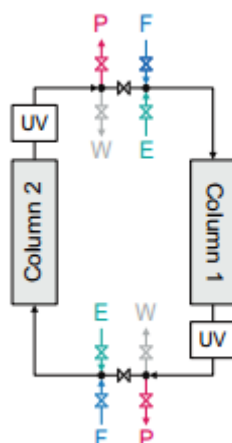


Figure 6 - Two-column, in open-loop, SEC purification step (P – Product; F – Feed; W – Waste; E – Eluent) [72]

These continuous purification strategies introduce a new range of possibilities and approaches to some, otherwise, difficult and expensive procedures. Different technologies have been introduced in comparison with batch chromatography and many research has been done reviewing both [73–75].

Products' purification complexity raises depending on the similarity between itself and impurities, resulting in increased elaborated methods and intricate applied technologies to solve the problem. When analysed through a chromatogram, starting with easier experiments, if products' peak demonstrates enough differentiation between its physical and chemical properties from impurities', a batch system could be enough. Nevertheless, continuous

processing can be considered depending on scale, propose or objective. Regarding more complex mixtures, namely binary or ternary, further laboured mechanisms have got to be taken into consideration, such as SMB or multicolumn solvent gradient purification (MCSGP) and gradient steady-state recycling (GSSR), respectively [76,77]. In fact, MCSGP has also been addressed as a purification method for biomolecules [78–80].

## 1.6 Modelling

The constantly evolving field of purification, and namely purification through chromatography is permanently improving or innovating in order to answer the increased demand for better, more efficient and robust strategies. The constant research and development (R&D) needed to ensure that this goal is achieved requires time, money and knowledge that are, in most case, short or applied elsewhere. So, the landscape presented itself for a descriptive, yet experimentally validated, approach that could please both worlds.

The R&D improvement was made through multiple attempts, in a trial-and-error system, adjusting distinct aspects and characteristics, eventually narrowing it down to the perfect ones that maximized intended objectives and complied with products' specifications. This process had many bottlenecks that started from being time-consuming, requiring expensive material and personal investments to the amount of adjustable parameters (buffer, salt column or pH [81]). With these problematics, conducting development and research using this strategy is hard, delayed and, far and for most, not viable.

The solution requires process' description through related equations of mass, flow, adsorption and others, that can reliably replicate what is or can be experimentally obtained. Using modelling as purification tool provides with impressive results while sparing precious time and resources. This approach allows for increased efficiency in development, robustness, economic viability, flexibility and system control [81].

Modelling based methods have been reported in association with a variety of purification strategies to some bioproducts, using, for instance, multicomponent or reverse-phase chromatography [86–88]. Regarding adenovirus, chromatography methods have been reported, described and optimized with computer's tools, focusing mainly on improving virus yields and column's productivity [7,41,69].

## 1.7 Analytical Methods

The overall process results and comparative studies depend on yields and removals calculated through established methods that evaluate a specific impurity or the actual and intended product. On bioprocesses concerning virus, the main impurities are host cell DNA and proteins. From the range of methods involved in the studied process, some were not only used for yield accounting but also for product characterization, due to their different applications or equipment's polyvalence.

Considering that every decision and conclusion about a strategy taken is laid upon established methods of analytical quantification, these are a keystone on process validation and execution. Their outcome can make or break a chosen strategy, justify novel approaches, enhance innovative studies or even discard a route taken. So, their validation must be taken seriously, as everything is depending on them. Multiple reviews have been done about this theme and method validation, considering the increased importance on food and drugs processes [85–87].

The process considered, reviewed and studied was quantified with the analytical methods showed at the following diagram (Fig. 7).

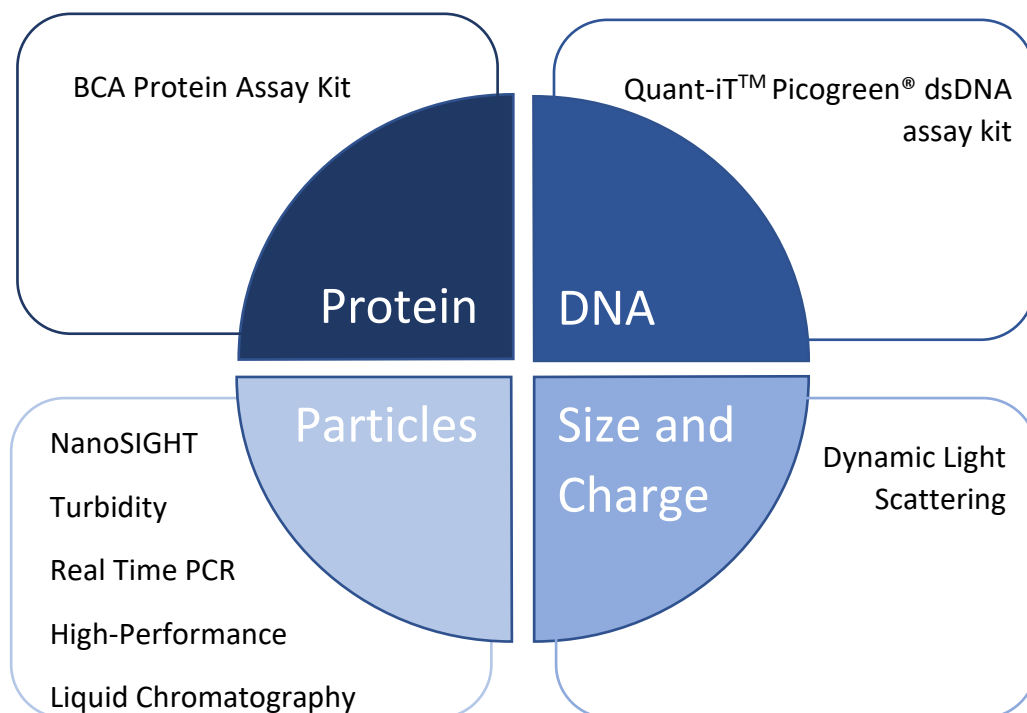


Figure 7 - Analytical Methods



# 2

# Methods and Materials

*This chapter describes each step of the downstream train and the analytical methods used for yield assessment and impurity removals.*



## 2.1 Virus production

A549 cells were amplified in T-flasks, then in HYPERflask® before being inoculated at a concentration of  $0.55 \times 10^6$  cell/ml. A 5 litres bioreactor was prepared for virus production. As operation parameters, the pH was settled at 7.2 and controlled by aeration with a gaseous  $\text{CO}_2$  and 1 M  $\text{NaHCO}_3$ , the dissolved oxygen was controlled at 40% air saturation by gas mixing with a temperature of 37 °C and gas flow of 0.2 L/min.

## 2.2 Clarification

The process started with a filter A, used to remove larger impurities, which has a pore size of 5  $\mu\text{m}$  and 0,2  $\text{m}^2$  of membrane surface area, followed by a second filter B with pores of 0,2  $\mu\text{m}$  and a membrane area of 0,1  $\text{m}^2$  to ensure maximum clarification.

Before starting, the filters were primed with 3 capsule volumes of water to remove preservative solutions and then equilibrated with working buffer (3 capsule volumes). For process monitoring, flowthrough and pressure overtime were measured. After each step, the effluent stream was sampled and analysed to evaluate process yields and removal ratios.

## 2.3 Ultra/Diafiltration

The clarified bulk was concentrated with a predefined strategy to ensure a certain virus concentration and impurity removal. The concentration was tested using tangential flow filtration (TFF) with Sartorius Slice 200 with a pore size of 300 kDa and an area of 200  $\text{cm}^2$ . The system was fed with a stable flow rate of 100 ml/min, using a Tandem 1081 Pump or SartoFlow Smart, both from Sartorius Stedim Biotech, established after adjusting previous trials results regarding protocol Shear factor (0.8 to 1 bar) and membrane surface area.

After correctly assembling the process, a primarily run was made with Mili-Q water to ensure perfect cleaning and total removal of preservative solution, followed by system equilibration with working buffer. To confirm correct transmembrane pressure (TMP) (Eq. 1), feed, retentate and permeate flows were monitored with polysulfone pressure transmitter

(Spectrum Laboratories, Inc., USA) connected to a Midject pressure display unit (Amersham Biosciences Corp., USA).

$$TMP = \frac{P_f - P_r}{2} - P_p \quad (1)$$

Permeate volumes were taken every few minutes with a technical scale (TE4101, Sartorius Stedim Biotech, Germany) connected to system pump. This methodology allows system calibration and throughout process monitorization in order to accomplish concentration factors and diafiltration volumes. After reaching desired final values, the TFF system was drained, with special enhancement on recovering virus retentate.

All process steps were conducted discontinuously which allowed sample collection from each flow and determination of overall yields and impurity removal ratios. When it was over, a complete sanitation was performed which consisted of multiples volumes of water and sodium hydroxide (0,5 M).

## 2.4 Purification (AEX)

The concentrated virus solution was purified by column chromatography in a bind-elute system using anion-exchange adsorbers. The chromatography trials were performed with different column mediums, including AEX resins (A and B) and a membrane.

After correctly assembling the column on ÄKTA explorer 10 liquid chromatography system (GE Healthcare, U.K.), a cleaning-in-place run was made to, once again, ensure total removal of preservative solution, followed by equilibration until stable UV signal. The chromatography runs were conducted at a flow rate 2.3 ml/min which corresponds to 300 cm/h of flow velocity.

Operation buffers were composed by 50 mM HEPES, pH 7,5 with 0 mM of NaCl for loading/running/washing or 2 M of NaCl for elution buffer. Before being used, each buffer was filtered with a 0,2 µm membrane filter (VacuCap® 90 PF Filter Unit). The ÄKTA system is associated with fractionator FRAC-950 (Amersham Biosciences Corp., USA), that allows precise and accurate fraction recovery after assessing correctly ÄKTA internal dead volumes.

## 2.5 Polishing (Size Exclusion Chromatography)

Purified viruses from the eluted pool were further treated using a size-exclusion chromatography column connected to the same ÄKTA system. The selected resin was packed inside an XK 16/20 column.

Due to the flow through working principles of a SEC column, only one buffer was used when polishing the adenovirus bulk, in this case, the HEPES solution without salt concentration. The column was loaded with a maximum of 20% of its volume and the runs were performed with a flow rate of 4 ml/min.

Identically to what was done after purification, a sample was taken from the polished virus pool to evaluate virus yields and the presence of impurities.

## 2.6 Analytical methods

### 2.6.1 Total protein quantification

The amount of proteins was quantified using BCA Protein Assay Kit (23227, Thermo Scientific, USA) according to the recommendations and manufacture's protocol. The calibration curve was established with bovine serum albumin (BSA) (23209, Thermo Scientific, USA). Predefined dilutions were applied in duplicate or triplicate, to ensure a measurement of average and standard deviation of each assay.

The absorbance was quantified at 562 nm on Infinite® PRO NanoQuant (Tecan, Switzerland) microplate multimode reader using a clear 96-well microplate (260895, Nunc, USA).

### 2.6.2 Total dsDNA Quantification

Using Quant-iT™ Picogreen® dsDNA assay kit (P7589, Invitrogen™, UK), the amount of total DNA was quantified. This fluorescent-based technique was done according to manufacturer's instructions and matrix interference was overtaken through successive dilutions (2-256 fold) with provided reaction buffer. Assay's results were obtained with Infinite® PRO

NanoQuant (Tecan, Switzerland) by measuring samples fluorescence in a flat transparent black 96-well microplate (3603, Corning, USA).

### 2.6.3 Nanoparticle Tracking Analysis

The concentration and size distribution of adenovirus was measured using NanoSIGHT NS500 (NanoSIGHT Ltd, UK). In this assay, each sample was diluted using D-PBS (14190-169, Gibco®, UK) to ensure that particle concentration was between the instrument's linear range, meaning from  $10^8$  to  $10^{10}$  particles per millilitre. All measurements were analysed with Nanoparticle Tracking Analysis (NTA) 2.3 Analytical software and performed at 23.3° C. The analysis is made through a 60-seconds video for each sample adjusting capture settings manually, namely shutter and gain, and considering particle size between 70 and 120 nanometres.

### 2.6.4 Turbidity analysis

Samples turbidity was obtained with 2100Qis Portable Turbidimeter (HACH, USA) in contrast with the standard provided. The instrument measures scattered light at a 90-degree angle from the incident light with a dilution of 1:3 applied to the sample. The results are obtained by directly applying the dilution factor to the displayed value. This instrument was selected for clarification assays because it measures heavily impure bulks that are otherwise unreliable to quantify.

### 2.6.5 Dynamic Light Scattering

Adenovirus' zeta potential and size were measured using ZetaSizer Nano Series (Malvern, UK) and Dynamic Light Scattering (DLS) technique. Each sample was diluted with one of two different buffers with a gradually increased pH value from 3 to 9. The cell used for each measure, DTS1060 (Malvern, UK), was previously cleaned with Mili-Q water and ethanol to clear remaining sample residues and equilibrated with next samples' buffer before being loaded with

it. All measurements followed standard procedure considering protein as samples and water as the dispersant, with 30 seconds of equilibration/optimization time. The analysis was done with ZetaSizer software 7.11 (Malvern, UK) at 25 degrees Celsius, considering a maximum amount of 100 measurements, but never less than 10 per run.

#### 2.6.6 Quantitative Real Time PCR

Virus DNA was extracted and purified with High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany) according to manufacturer's instructions after previous digestion of viral suspension with DNase following standard procedures. Genome containing particles were measured by real time quantitative PCR (q-PCR) using respective protocol and LightCycler 480 (Roche, Germany).

#### 2.6.7 High-Performance Liquid Chromatography

The High-Performance Liquid Chromatography (HPLC) method using a column with size-exclusion resin was introduced to evaluate sample content, concerning both virus and impurity concentrations. The TSKgel G5000PW column (TOSOH Biosciences, Japan) was connected to the ÄKTA system described above or to the WATERS 2695 Separation Module (Waters, USA).

When connected to ÄKTA system, a 100 µl loading loop and a constant flow of 1 ml/min were used, contrasting to the multiple vial system on the separation model which used volumes of 50 µl per sample, running at 0,7 ml/min. The second equipment works for itself after method definition and period description, allowing overnight runs. In both cases, samples were injected in flowthrough mechanism, into a pre-equilibrated column with running buffer (50 mM HEPES, pH 7,5 with 200 mM of NaCl). After several injections, a cleaning-in-place was applied in both upper and down flow, to ensure maximum impurity removal, which included multiple column volumes of water (Mili-Q) and NaOH (1 M).





# 3

## Implementation and Development of an analytical tool for process monitoring

*The following chapter explains the implementation of an orthogonal analytical method for particle tracking that brings together easy procedures with accurate measurements.*



### 3.1 The need for reliable analytical methods

The uprising specification complexity and narrowing on the quantity of impurities allowed in every step of the process increases the research for reliable and robust analytical methods that can provide real time data in the most efficient way. Alternatively, for product control on a specific process step, there is a need for a tracking assay that could present trustworthy results while requiring minimum operator dependence and ensuring throughout precision and accuracy.

In order to achieve it, an orthogonal method was tested and calibrated with the product, which made use of two previous presented methods, providing distinct types of data that can be easily correlated through a calibration curve. Even though the pore size of the SEC column guarantees that the viruses elute in the porous volume, a NanoSIGHT tracking analysis ensures that the measurement obtained doesn't represent aggregates and other particles. The respective values obtained by NanoSIGHT NS500 (NanoSIGHT Ltd, UK) and High-Performance Liquid Chromatography (HPLC-SEC) were associated and a thorough analysis of each method to ensured maximum applicability and will be discussed further on.

The appealing factor of this approaches comes from being an alternative for virus quantification different than qPCR which is expensive and time-consuming, requiring simpler procedures with the same reliable results.

### 3.2 NanoSIGHT Calibration Curve

The correct use of this technology depends on preselected dilution to ensure complete analysis of the spectrum present to fully account for the number of particles. The obtained results present a distribution curve from 0 to 1000 nm of the analysed particles and an estimation of concentration is done for an interest range (in this case, from 70 to 120 nanometres) (Fig. 8). Although useful for establishing the presence and number of particles, its results are, in a way, too much dependent on technician's adjustments to ensure reliability and robustness on a single measurement so at least three must be made.

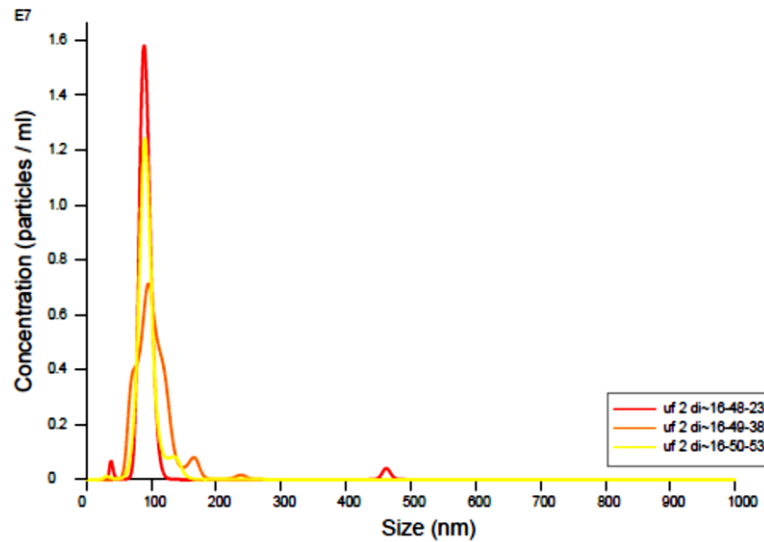


Figure 8 - NanoSIGHT assay with 3 measurements

NanoSIGHT was evaluated on a TFF concentration and diafiltration process, with results being reported below (Fig. 9). These steps include a two-fold concentration and four times diafiltration for buffer exchange. The results were obtained after 3 to 5 replicates of each sample to increase precision.

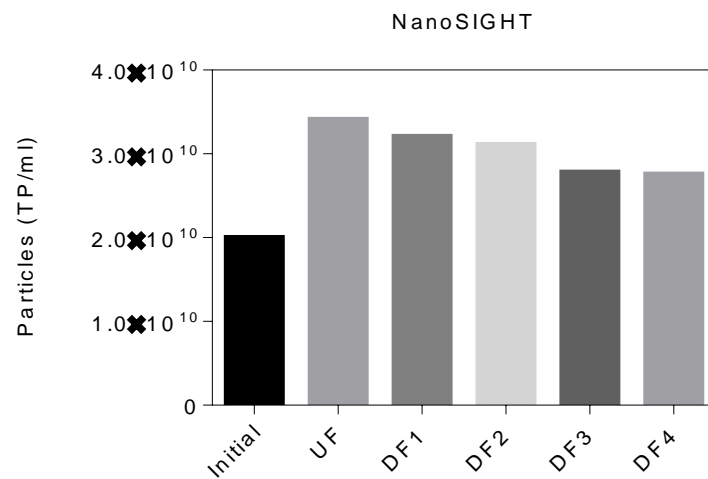


Figure 9 - NanoSIGHT assay for Ultra/Diafiltration

Even though it decreases productivity and consumes valuable time, this assay must be considered, as its outcome is most valued for process monitoring.

Taken that into account, an experiment was done with the diafiltrated product in order to establish a relation between particle concentration and dilution factor for further method implementation in correlation with HPLC-SEC. The product was diluted as far as 10 times, and the initial concentration was measured 5 times to ensure maximum accuracy. The results show that a tendency line with almost 0.93 of correlation factor (Fig. 10) is achieved and an association can be made with another method for the same dilutions applied.

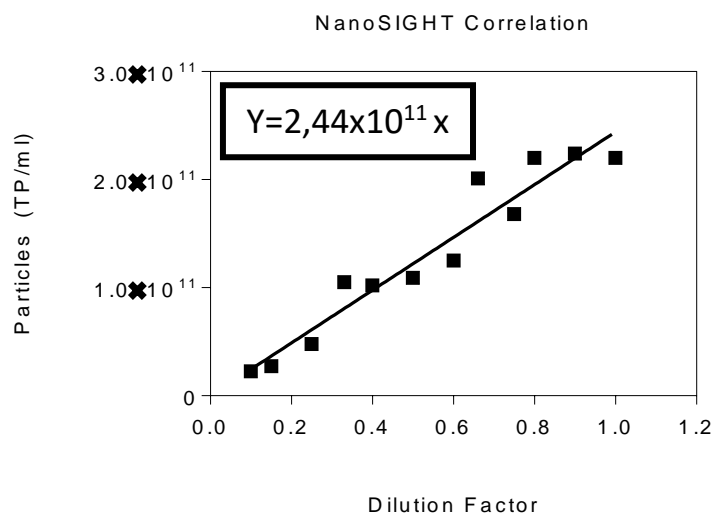


Figure 10 - NanoSIGHT Correlation

### 3.3 HPLC-SEC Calibration Curve

The application of HPLC as an analytical method introduces great reliability and allows for impartial and intemperate results as the outcome doesn't rely on technician's adjustments.

The use of a size-exclusion column (TSKgel G5000PW) ensures that the viruses are not caught on the matrix, as it's demonstrated by Figure 3, and produces a simple chromatogram that shows the magnitude of its present species in the mixture as it flows through the column. According to the expected concentration of the sample, the injected amount can be optimized to ensure that a proficient reading is obtained. Besides relation between presence and peak intensity, different wavelengths can be selected to monitor different biomolecules.

As was done before, another TFF process was monitored by HPLC-SEC after clarification (Fig. 11). The results were obtained with injections of 100 microliters allowing for complete flowthrough before the next sample is introduced.

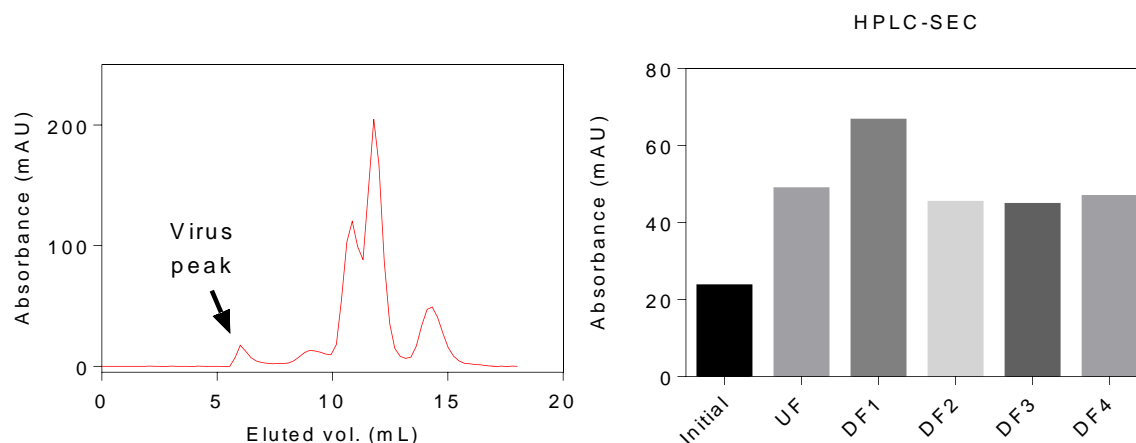


Figure 11 - HPLC-SEC assay for Ultra/Diafiltration (wavelength 280 nm)

With this approach, the interpretation and data analysis between steps is direct and easily understandable, as the volume injected is the same, the peaks magnitude depends only on species presence. As mentioned before, these peaks were fractionated and analysed with NanoSIGHT to ensure that corresponded to single virus and not aggregates. For the presented flowthrough intensities, a wavelength of 280 nanometres was used as it is the most common to trace proteins.

In order to make sure that the obtained intensities are to be taken seriously, a study was made correlating known dilutions and chromatography monitoring. This approach started upon several repetitions of the initial concentration and going until 5% of this value.

Later on, to evaluate the upper limits of the quantification the initial sample was concentrated 2 and 5 times (Fig. 12). As mentioned above, the 5-fold concentration required a smaller injection to be made and only 50 microliters were loaded. The calculations for this procedure are direct, as half of volume injected corresponds to half intensity detected.

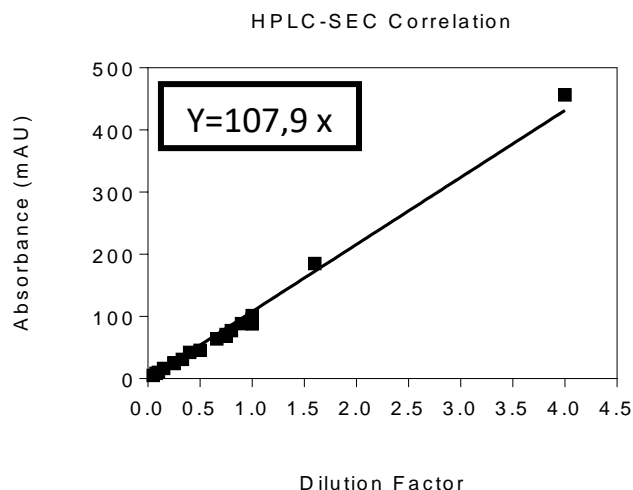


Figure 12 - HPLC-SEC Correlation (wavelength 280 nm)

This chart illustrates that the obtained results from HPLC are robust and reliable, with concentration and dilution factor exhibiting a linear dependence. The data points were obtained by integration of the chromatogram in UNICORN. The results have a 0.99 correlation factor to the trendline which describes them.

### 3.4 Combined approach

By combining these two methods it is possible to ease the task of process monitoring and facilitates tracking of yields and particle accountability in every step.

As it's demonstrated by the correlation factor, or standard deviation, on the represented trending lines, the HPLC produces a much more accurate group of measurements. This fact combined with its direct and untampered procedure, cement it as a great analytical method for bioprocesses.

The interest in associating these methods comes from the requirement of establishing yields and meeting specifications that are accounted in particles present on a certain volume. As NanoSIGHT provides it with relatively low precision, the HPLC can complement it and facilitate the acquiring procedure. In order to achieve it, a calibrated correlation has to be established by several analyses on both systems.

The method used for this calibration relied on introducing the same samples in both types of equipment and correlating them until a certain degree of concentration or dilution.

Meaning that in higher or lower concentration, adjustments were made in each assay to ensure correct measurements. Furthermore, to confirm method robustness some samples were introduced several times, in particular, the initial concentration obtained from the filtration processes. The individual data was ranged for both validation limits to ensure that the correlation chart had maximum compatibility (Fig. 13 a, b, c).

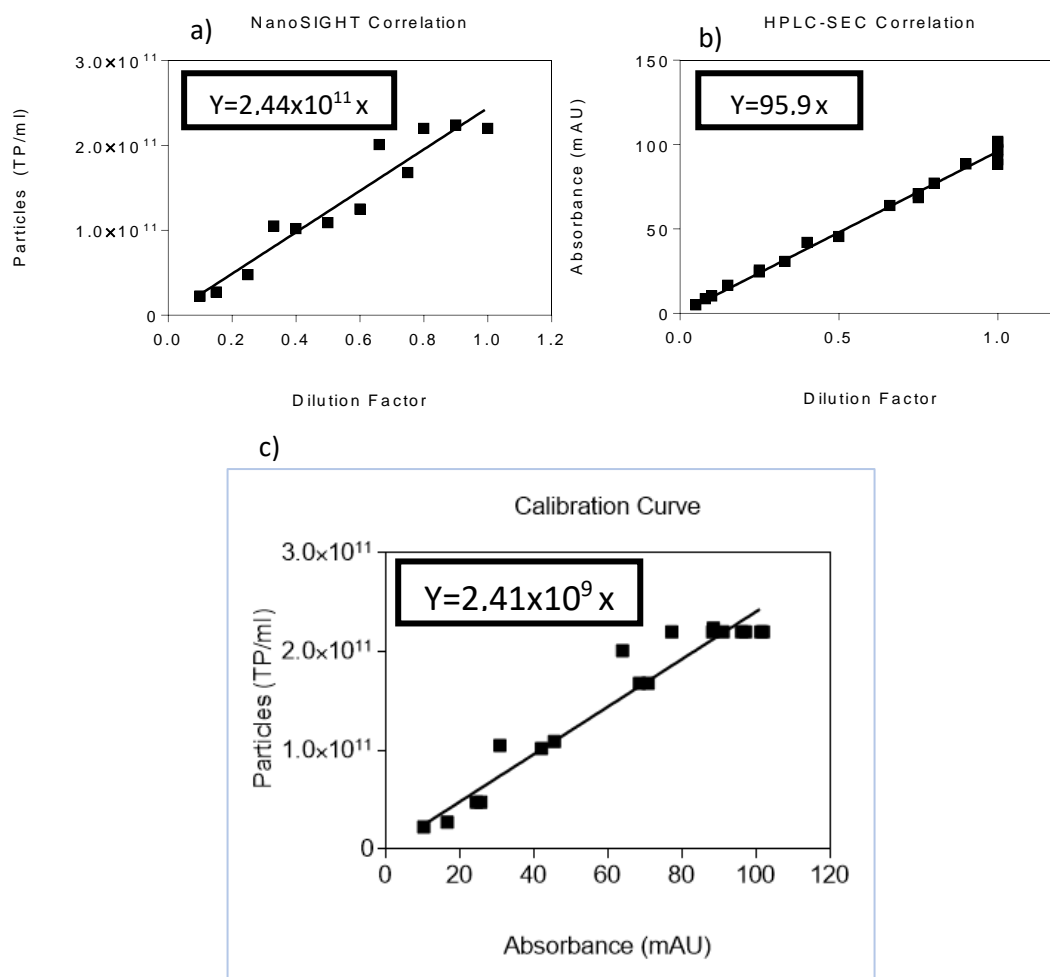


Figure 13 - NanoSIGHT correlation for design range (a),  
HPLC-SEC correlation for design range(wavelength 280 nm) (b), Correlation curve (c)

The obtained trendline presents a correlation factor of 0.94, which makes for a reasonable tool for process monitoring. Considering this approach is possible to evaluate each step taken by introducing the respective samples on the equipment overnight and access to progress made without spending valuable time and expensive resources.



### 3.4.1 Detection Limits and Errors

The validation limits of a method establish the range of its applicability by determining where the results obtained are a viable measurement of reality. These parameters include the determination of minimum and maximum limits and the error associated with the tendency line that describes the evolution of the experience.

The minimum detectability (D) is a parameter which includes a determination of equipment' sensibility (S) and amplitude of noise taken through the assay (N). The first is calculated with the peak obtained (E) by introducing a previously characterized sample with known concentration (C<sub>i</sub>) (Eq. 2). The noise amplitude (N) is a result of absorbance at the baseline within a time frame (Fig. 14). With these determined, the minimum detection limit was calculated at  $4,7 \cdot 10^9$  particles per millilitre which correspond to 1,95 mAU when using the HPLC-SEC assay (Eq. 3).

$$S = \frac{E}{C_i} \quad (2)$$

$$D = \frac{2N}{S} \quad (3)$$

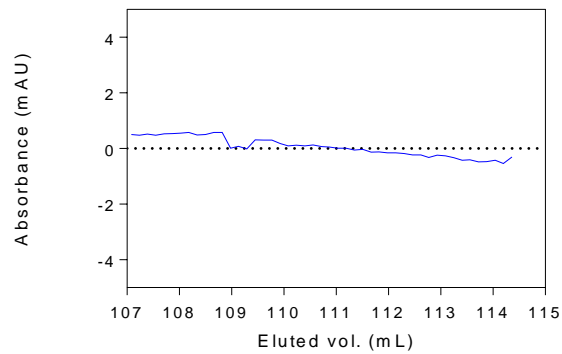


Figure 14 - Noise amplitude at the baseline  
(wavelength 280 nm)

The maximum particle concentration from which linearity isn't verified was determined with the correlation curve and the attachment of specific new lines (Fig. 15). These new ones correspond to a deviation of 5 % to the line slope and establish the limit from where the measurements are not trustworthy. With the charts analysis, it's concluded that the maximum amount of particles per millilitre correctly detected by the method is  $2.32 \cdot 10^{11}$  or 96.1 mAU.

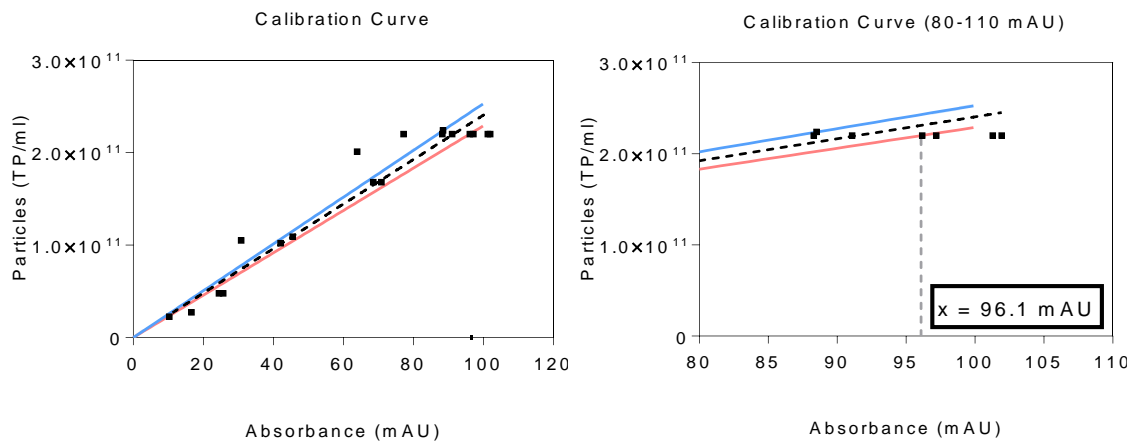


Figure 15 - Maximum particle concentration acceptable, right figure is focused on the 80 – 110 mAu region of the left plot

After completing the limitation ranges, it's relevant to establish error margins to correctly express a measurement of quantity. These boundaries were calculated with the tending line and experimental data, obtaining a value that induces certainty to experimental results. With this in consideration, it's possible to conclude that from 1,95 to 96,1 mAU, the number of particles per millilitre can be calculated with an error of  $8,2 \times 10^9$  TP/ml. This margin can have different significance depending on the stage of the process. For instance, clarified bulks have around  $10^{10}$  TP/ml which makes the error being close to 20%. For the following steps, with the increased concentrations, its repercussions on the measurements are less than 10%.

# 4

## Results and Discussion

*This chapter presents the outcome of each assay taken throughout the downstream process and the considerations made to access the best conditions and parameters for adenovirus purification.*



## 4.1 Development of a purification process for oncolytic virus

The optimization and development are the focus on research studies applied to downstream as its improvement is directly related to overall applicability and most important, viability. Depending on the knowledge that precedes the intended product, the focus on development could be differentiated between a completed analysis of each step needed to achieve planned goals or directing all efforts to optimize a particular step for maximum productivity.

The implemented purification process for oncolytic viruses englobes a series of increasing complexity steps that require a wide variety of means and induce the product to several environment changes (Fig. 16). Nevertheless, the acceptance and recognition of this strategy are relentless proven by the reported yields obtained from it when applied to different oncolytic viruses. These results justify that for recently discovered oncolytic viruses, this is the fall-back procedure used for purification. With more research and as their behaviour in different conditions is known, the overall process with the focus on downstream can be improved with recently developed strategies for each step.

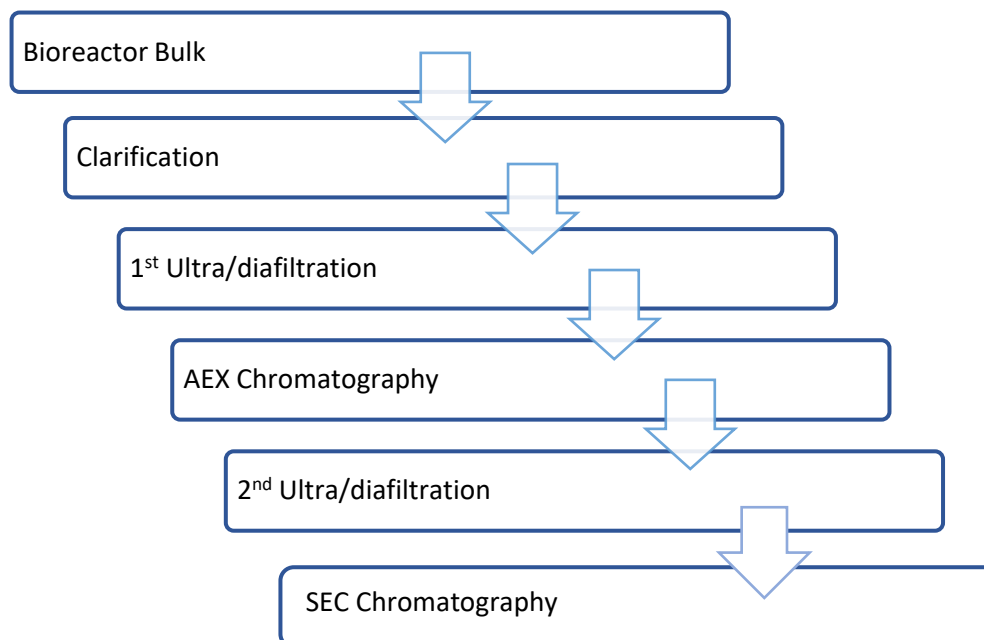


Figure 16 - Standard downstream processing train for adenovirus purification [7]

#### 4.1.1 Clarification studies

After bioreactor production and cell lysis, the adenovirus bulk initiates the downstream processing train with clarification. The focus on this step is the removal of larger impurities like cell debris. In order to accomplish it, a 2-step filtration strategy was tested as previously mentioned. The decreasing of pore size ensures maximum impurity removal while controlling pressure and folding layer issues.

Before initiating a step clarification, the depth-filter must be cleansed with equilibration buffer to ensure complete removal of the preservative agent. After this stage, some measurements of flux are taken at different flow rates (120, 200 and 333 ml min<sup>-1</sup>) for both filters to determine the maximum achieved. These assessments are made only with buffer as maximum flux is only obtained when the membrane isn't obstructed (Table 3). At the same time, the pressure increment was tested for each named flux to establish a baseline to use at clarification (Fig. 17).

Table 3 - Water Fluxes for different filters: (A) and (B) calculated from observed Flow rates

	<b>Filter A (5 µm pore, 0,2 m<sup>2</sup>)</b>		
<b>Flow Rate (ml.min<sup>-1</sup>)</b>	110	197	319
<b>Area (m<sup>2</sup>)</b>	0.2		
<b>Flux (L.min<sup>-1</sup>m<sup>2</sup>)</b>	33	59	96

	<b>Filter B (0,2 µm pore, 0,1 m<sup>2</sup>)</b>		
<b>Flow Rate (ml.min<sup>-1</sup>)</b>	99	196	325
<b>Area (m<sup>2</sup>)</b>	0.1		
<b>Flux (L.min<sup>-1</sup>m<sup>2</sup>)</b>	59	117	195

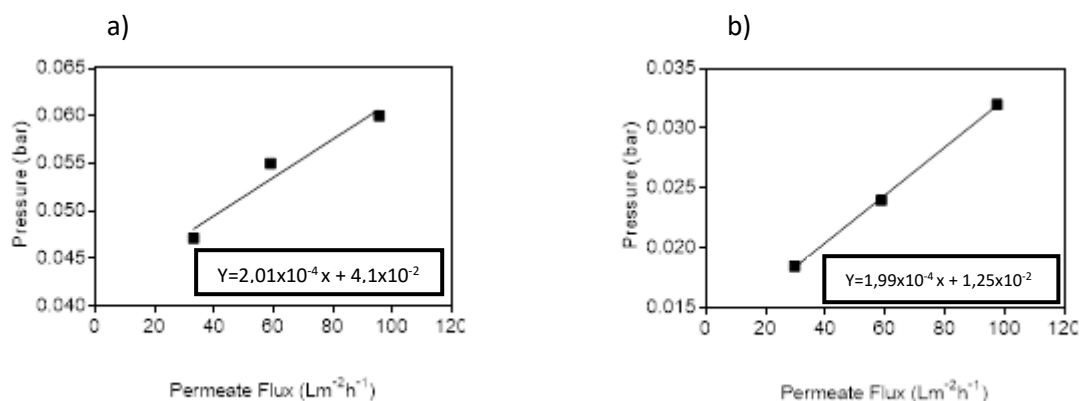


Figure 17 – Pressure variation for different filters: A (a), B (b) for observed water fluxes

The increasing pressure observed along the procedure is caused by the cumulative pore clogging (Fig. 18), this result justifies the decrease of flux observed despite the positive displacement of the pump.

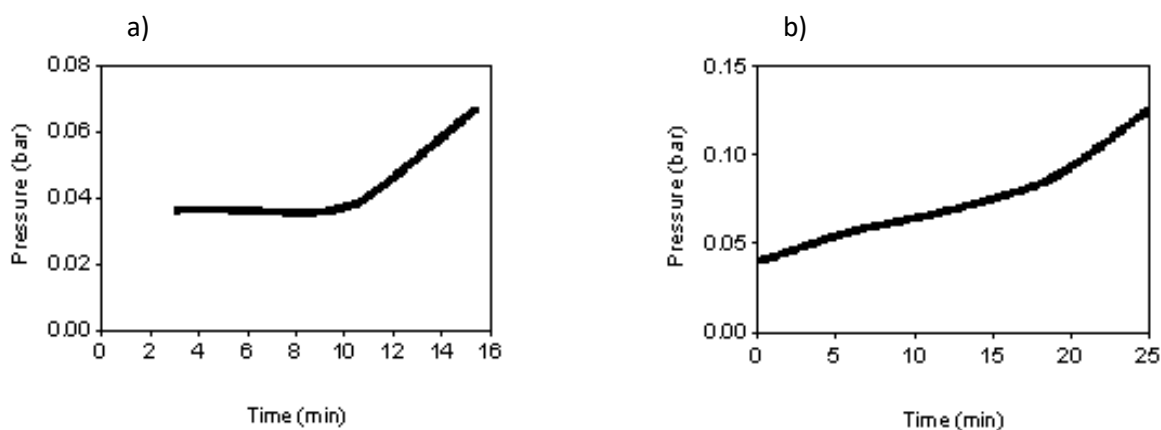


Figure 18 - Pressure over time: A with a pore size of 5 μm (a), B with pores of 0,2 μm (b)

The clarification results were assessed with NanoSIGHT and turbidity assays. The outcomes prove the previous runs made and demonstrate the efficiency of filters and strategy chosen. The turbidity analysis proves the clearance of a sample in comparison with an established reference and as wanted, the initial sample is much denser than the clarified one (Fig. 19 a). Instead of using the implemented HPLC-SEC method, due to impurity content at this stage, a direct NanoSIGHT assay was performed to evaluate the number of particles present and the yield of clarification achieved with this 2-step strategy (Fig. 19 b). The results obtained with

the turbidimeter show a 73% decrease of product turbidity as was expected. The total particle count from NanoSIGHT reveal that no viruses were lost during the process.

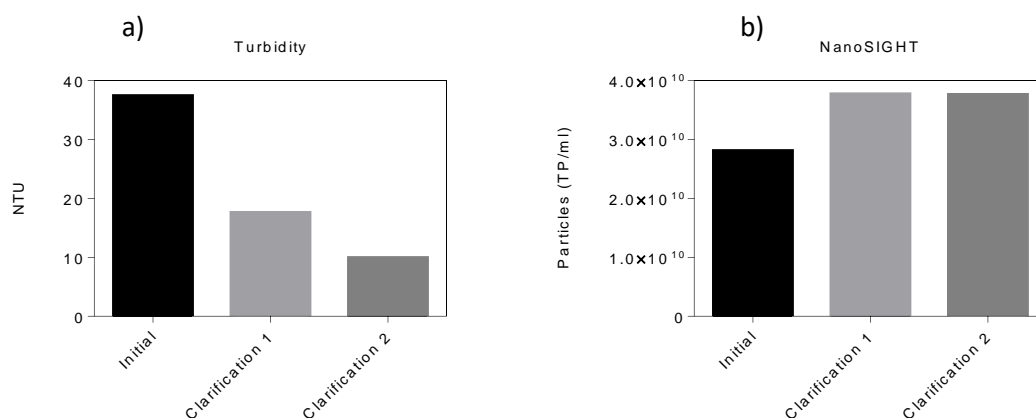


Figure 19 - Results of clarification: Turbidity (a) and NanoSIGHT (b)

These outcomes justify the strategy taken to the product handled. The reduction of turbidity to levels below 10 NTU and the high recovery of total particles demonstrate that this 2-step filtration approach is viable to clarify these oncolytic viruses. For the following steps, a reduction in impurity content is much relevant because the continuous decrement in pore size would be endangered by clogging.

#### 4.1.2 Ultrafiltration and Diafiltration Steps

After clarification, and before being suitable for purification, the oncolytic viruses were ultra/diafiltrated. This process has gained a lot of attention as many new strategies could be applied depending on the virus. The previous research made indicated that the optimal procedure to impurity removal and virus concentration was a two times ultrafiltration followed by 4 diafiltrations with equilibration buffer.

A cassette membrane filter with  $200 \text{ cm}^2$  and a 300 kDa cutoff was evaluated. The cassette was previously equilibrated with buffer and through the entire procedure the permeate flux was monitored to determine its evolution overtime with increasing pore clogging (Fig. 20).



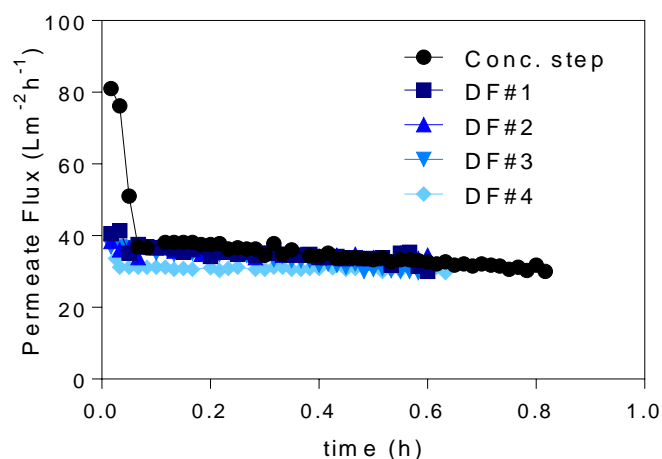


Figure 20 - Flux in Ultra/diafiltration for a cassette membrane filter cartridge with 200 cm<sup>2</sup>

The results obtained by this analysis show that a greater permeate flux of almost 40 Lm<sup>2</sup>h<sup>-1</sup> is obtained, this might be associated to the different flow regime provide by this device, whereas in the hollow fibre the flow is usually laminar, that is not the case in flat sheet cassettes where usually the flow is turbulent thus delaying pore clogging.

The process yield was evaluated with the NanoSIGHT-HPLC method explained before with its results presented in the following table (Table 4).

Table 4 - Ultra/diafiltration results for Membrane Filter

	Initial Concentration	UltraFiltration	Diafiltration			
			1	2	3	4
mAU	18.489	44.898	44.961	47.573	56.428	53.603
TP/ml	4.46E+10	1.08E+11	1.08E+11	1.15E+11	1.36E+11	1.29E+11
Yield						
Overall	104%					

Through this approach, a 104 % yield of purification after 4 diafiltrations was obtained, which is reasonable for the error margin considered. These results from HPLC analysis and analytical methods show a similar impurity removal as was obtained when using the hollow fibre

cartridge. With a comparative analysis is also concluded that the virus peak is more intense than the present impurities (Fig. 21).

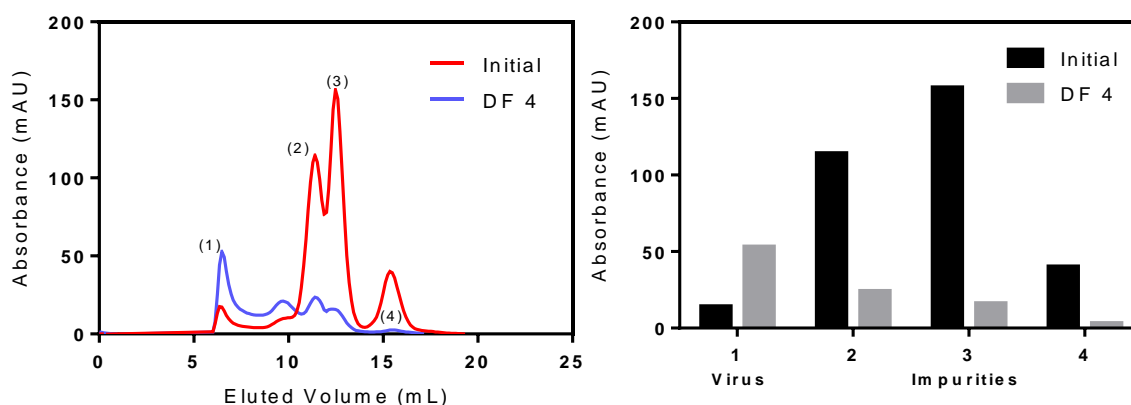


Figure 21 - HPLC-SEC comparing product and impurities (wavelength 280 nm) using Membrane filter

With the analysis of both procedures, it's concluded that either strategy deliver satisfactory and reliable results on what concerns impurity removal but majorly increment on virus concentration. Depending on the scale, and bulk volume, each approach has its applicability. These conclusions regard specific biomolecules, for instance, the absence of envelope on adenovirus makes them capable to endure higher shear rates which allows processes with greater fluxes. From this conclusion, when a small batch is needed for scouting, the hollow fibre can be a reasonable solution. To larger assays, and scale up processes, the membrane cassette presents itself as a more suitable approach by allowing greater flow rates which diminish overall process length while maintaining quality specifications. Another advantage of this technology is the possibility to connect it to an automated equipment (Sartoflow Smart, Sartorius) which performs ultra and diafiltration by itself, controlling the intended pressures and flow rates determined by scouting, and even offering the possibility to perform continuous diafiltrations.

#### 4.1.3 Chromatography purification

The chromatography strategies developed to purifying this oncolytic virus focused on several possibilities, from different resins and membranes to elution gradients or steps with different salt concentrations.

Before starting chromatography experiments a surface potential measurement was conducted to evaluate the oncolytic virus charge in solutions with different pH and determine its isoelectric point (Fig. 22). This analysis concludes that the isoelectric point is not achieved at the range of pH handled, which narrows the ligands and resins that were possibilities for purification. Despite maintaining a negative potential thorough the range of pH's tested, lower values represent acid and dangerous environments for virus stability. When close to 0 mV, parallelly to the severe environment for adenovirus, the lack of repulsive charges or forces induces virus precipitation and therefore their loss. By maintaining the pH range from 7 to 8, virus stability is ensured throughout the entire process. As the negative potential is preserved within this interval, the anion-exchange chromatography presents itself as a suitable purification approach.

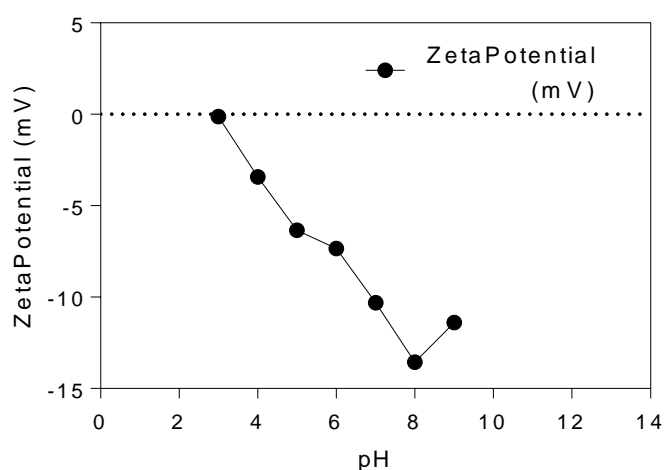


Figure 22 – Zeta Potential for adenovirus

With these considerations, the first strategy consisted of using a membrane adsorber. This is a ReadyToProcess membrane device with ligand Q, that allows good impurity removals. The chromatography runs taken were conducted at  $5 \text{ ml min}^{-1}$  and different injections were made to establish the 10% of virus breakthrough. The outcome was evaluated by Nanoparticle tracking analysis (NTA) using NanoSIGHT, RT-qPCR and impurity analytical methods.

As a first attempt, 50 ml of diafiltrated bulk were injected and with this, no breakthrough was achieved (Fig 23 a). This fact was evidenced by NTA which only detected 5% of feed material in flowthrough. In result, another effort was done to achieve the intended objective. This time an increase of 5 times of the previous amount was injected and the breakthrough was obtained

after more than 100 ml of virus bulk (Fig 23 b). The result was evaluated with RT-qPCR and the establishment of  $C/C_{\text{feed}}$ . The  $\text{DBC}^{10\%}$  measured was  $2.7 \times 10^{12}$  VG copies  $\text{ml}^{-1}$  of membrane and the impurity detection methods showed relevant removals of around 80% for host cell DNA and total protein. Concerning virus recuperation, it was obtained a 78% yield after elution with 2 M of salt concentration.

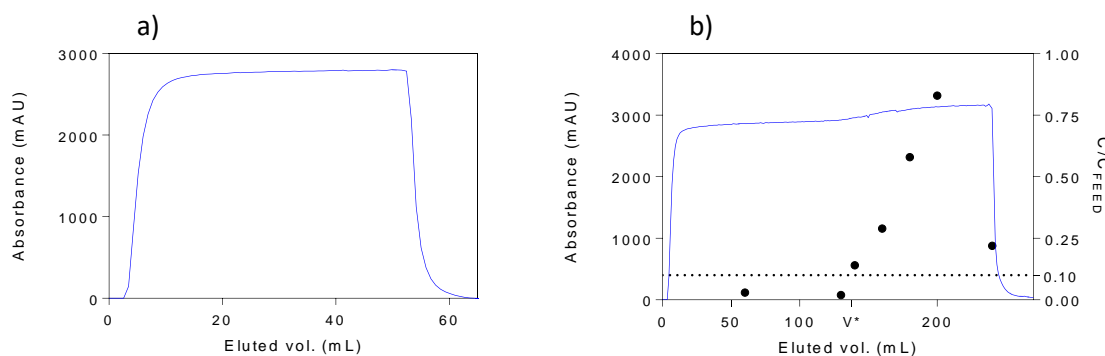


Figure 23 -  $\text{DBC}^{10\%}$  of membrane adsorber (wavelength 280 nm): 50 ml injection (a) and 250 ml injection (b)

Although these results are promising for oncolytic viruses, the overall spectrum of possibilities tested showed a greater match. Nevertheless, membrane chromatography presents the possibility of volume reduction and purification in a single step while providing good scale up solutions that could make this a viable solution for other applications.

The first condition verified was the flowrate used for sample loading. The ones tested were  $1.6 \text{ ml min}^{-1}$  and  $2.3 \text{ ml min}^{-1}$ , which considering that the column used has a 5 ml packed bed volume, corresponds to 200 and 300  $\text{cm.h}^{-1}$ , respectively. In these chromatography runs, the viruses were eluted with the same elution buffer used in Sartobind Q experiments. From the results obtained, when comparing the  $\text{DBC}^{10\%}$  from the first run ( $2.81 \times 10^{11}$  TP/ml) with the second ( $1.7 \times 10^{11}$  TP/ml), it is possible to conclude that there is a dependence of dynamic binding capacity with the flow-rate. This is somewhat expected since we are dealing with a resin based adsorber in contrast with a continuous and convective media such as membranes. An example of a chromatogram obtained with this media is shown in Figure 28.

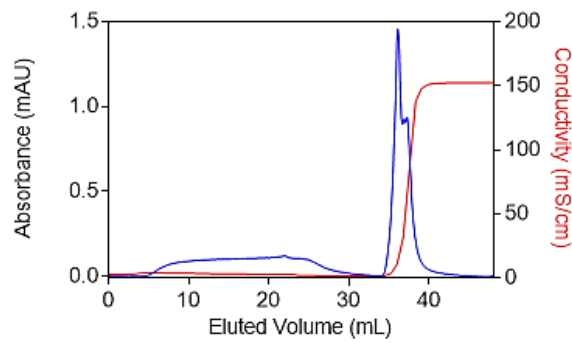


Figure 24 - Chromatogram using AEX media A (wavelength 280 nm)

After particle tracking analysis, host cell DNA and total protein removal yields were obtained. These shown valuable progress towards purification as 94% and 95% of respective impurities were removed. For this case, a 74% virus recovery was obtained after elution.

The same experiment was conducted with the AEX resin B. In this case, the flowrates applied were 2.3 and 3.2 ml.min<sup>-1</sup>, which correspond to 300 and 400 cm.h<sup>-1</sup>. The entire assay was monitored by absorbance using a wavelength of 280 nm (Fig. 25).

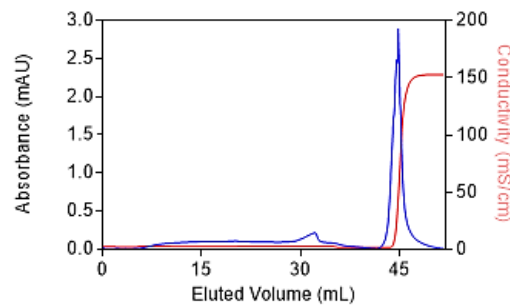


Figure 25 – Chromatogram using AEX B (wavelength 280 nm)

After analysing the  $C/C_{\text{feed}}$  over time, a dynamic binding capacity at 10% was determined for each flow velocity, resulting in  $6.2 \times 10^{11}$  and  $2.7 \times 10^{11}$  TP/ml for 300 and 400 cm h<sup>-1</sup>, respectively. The impurity removal ratios were inferior than before, but still promising for this application. The host cell DNA content was lowered to 85% and total protein for 90%. A major factor on this column is the virus yield recovery achieved, which was 85%.

Although apparent benefits of using membranes adsorbers, the selected media for further process development was AEX B. This media presents a virus recovery of 85% and considerable impurity removal ratios (Table 5).

Table 5 - Chromatography comparative study

Chromatography Media	DBC <sup>10%</sup> (TP/ml)	Virus Recovery (%)	DNA removal (%)	Total protein removal (%)
AEX membrane	2.7x10 <sup>12</sup>	78	80	80
AEX Resin A	1.7x10 <sup>11</sup>	74	94	95
AEX Resin B	6.2x10 <sup>11</sup>	85	85	90

With the decision of the chromatographic media, the focus changed to salt concentration and its repercussions in virus recovery and impurity removal. As mentioned before, a salt concentration is used to destabilise the equilibrium created inside the column while the loading takes place and elute the purified virus. The elution recoveries of the previous described experiments were obtained through a 2 M salt concentration elution step. Although a high recovery can be obtained the excess strength of elution might counteract the purification process by over eluting absorbed impurities, or affect products stability and therefore viability.

This aspect was further evaluated in two different routes. Firstly, by pursuing the minimum amount of salt concentration needed for full virus recovery with fewer impurities desorbed. And secondly, by performing a first wash step with a low salt concentration prior to elution that would not affect virus adsorption but would greatly improve the impurity removal ratios obtained.

As a mean to accomplish it, and considering the previously DBC obtained data, the AEX B was repeatedly loaded with calculated volumes. After completing the washing procedure, a series of continuously increasing salt concentration gradients were introduced in the column (Fig. 26 a, b, c, d, e).

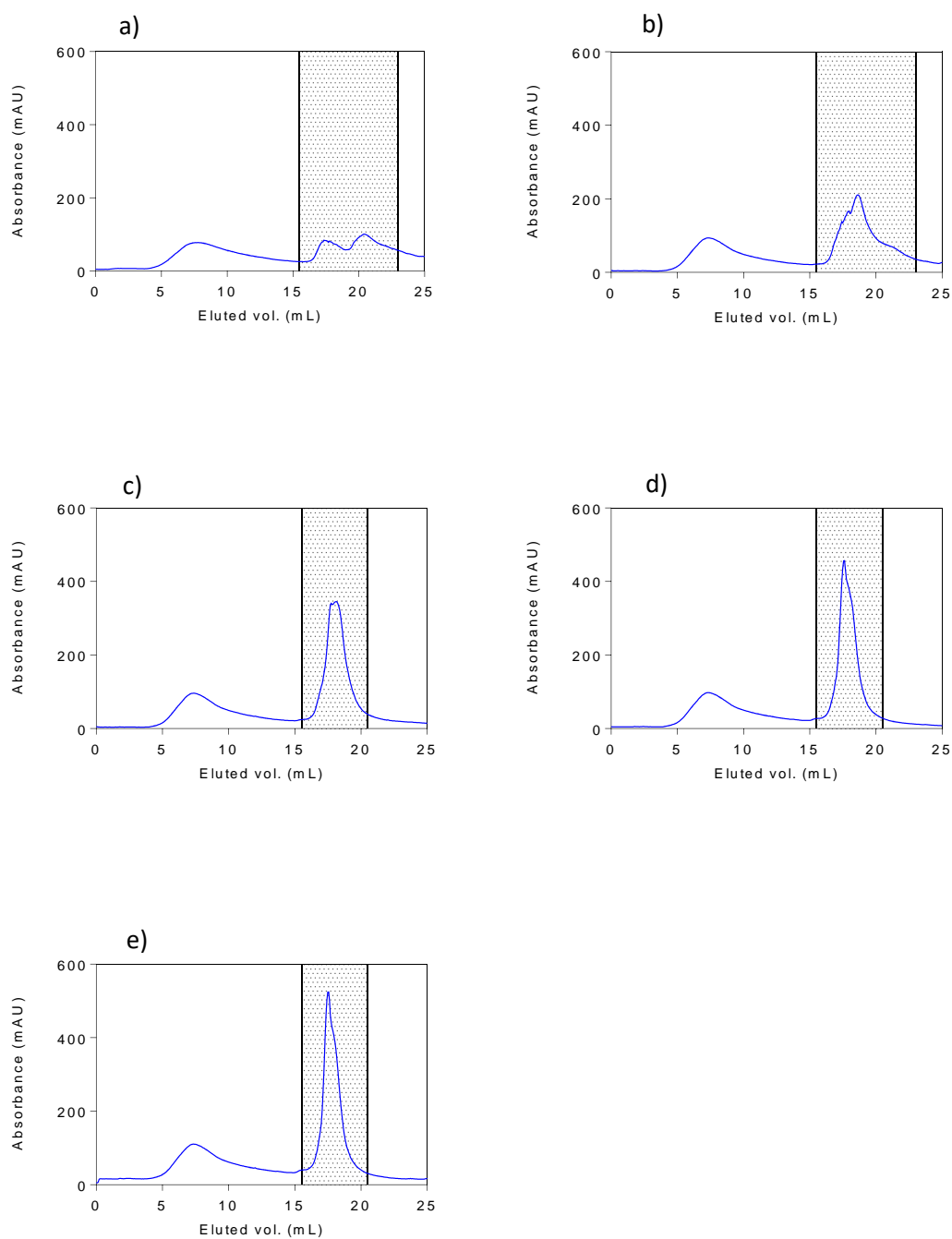


Figure 26 - Elution experiment on AEX resin B (wavelength 280 nm) for 150 (a), 487 (b), 825 (c), 1162 (d) and 1500 (e) mM

The elution steps included a minimum of 150 mM of salt concentration increasing until 1.5 M with other 3 stages in between. The maximum concentration was lowered from 2 M due to previous research and knowledge of the elution step.

The result of this experiment was analysed by NTA to determine the quantity of eluted particles, and DNA Picogreen assay and BCA protein method to verify the eluted impurities in each step. The outcome showed valuable improvements on impurity removal and proved that the lower concentration clearance steps can enhance the purification outcome of higher elutions (Fig. 27). The removal ratios verify that small concentrations can achieve an 80% reduction in impurities per millilitre of sample.

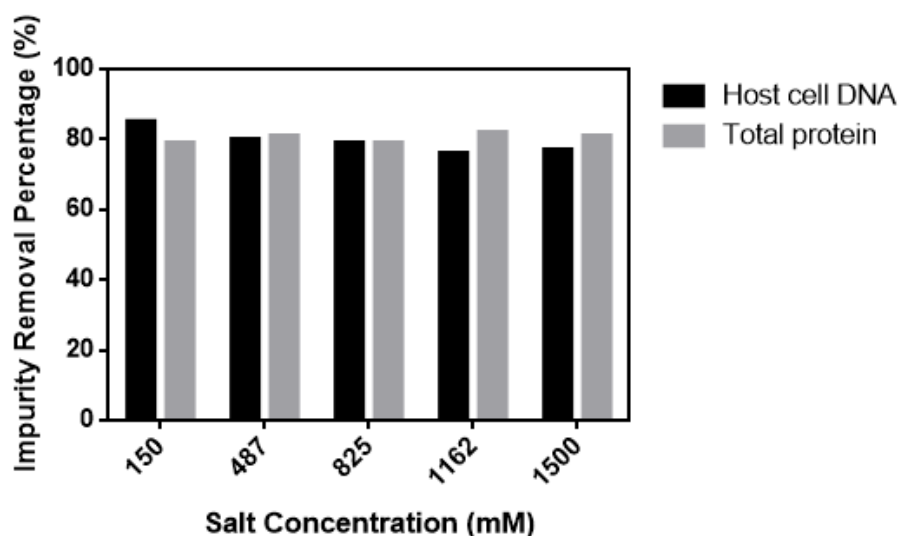


Figure 27 - Impurity removal ratios for different salt concentration elutions

This accomplishment introduces a new feature to the purification procedure that can greatly improve the process outcome by previously removing a relevant amount of impurities. To evaluate applicability, a particle tracking analysis was done to verify its effect on virus recovery in smaller gradient intervals. The results demonstrate that the small gradients applied didn't elute a significant amount of virus particles when comparing to the great improvement on contaminants' removal (Fig. 28). And so, this step could be introduced in chromatography purification to improve quality without compromising recovery.



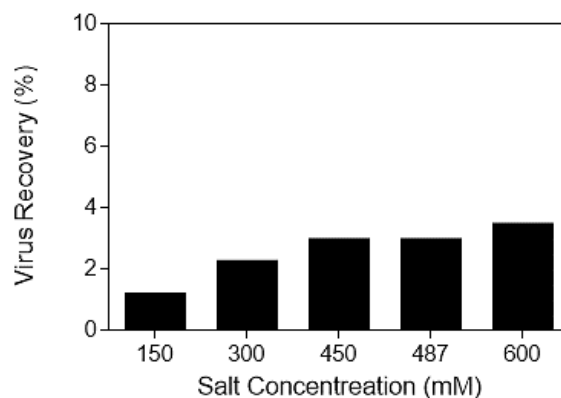


Figure 28 – Virus recovery ratios for low salt concentration elutions

At the other end, the experiments focusing virus elution demonstrated that concentrations higher than 825 mM would elute a significant amount of adsorbed virus. This fact is relevant to process planning when the virus stability is a concern (Fig. 29). Another process would require higher concentrations and even reach 2 M, which may demand quick handling for ensuring quality preservation.

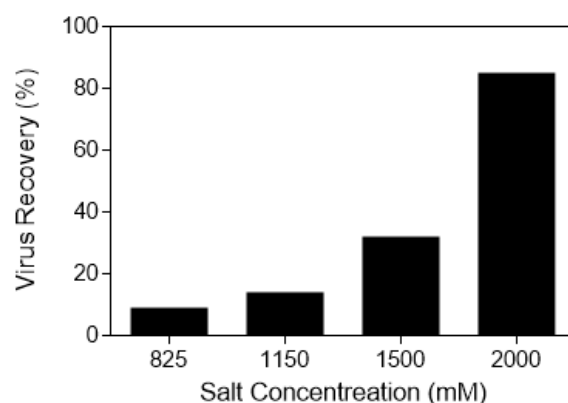


Figure 29 - Virus recovery ratios for high salt concentration elutions

With the data obtained, column conditions and process parameters were defined for batch chromatography. In resume, it was established that to maximize productivity and quality, the column loading volume would be limited to  $6.2 \times 10^{11}$  particles per resin millilitre, with a loading flow rate of  $2.3 \text{ ml min}^{-1}$  or  $300 \text{ cm h}^{-1}$  and virus elution would be preceded by a small salt concentration gradient of 200 mM to remove absorbed impurities and considerably improve products purity.

After these trials, a final validation run was performed focusing completely on virus recovery. These consisted of a pair of chromatography comparative runs differing in NaCl concentration on the sample to be loaded (0 and 200 mM) (Fig. 30 a, b). The eluted product was evaluated by HPLC-SEC method and shows that the result of the 200 mM experiment has a higher concentration ( $9.5 \times 10^{10}$  particles/millilitre) than the one from the run without NaCl in the loaded sample ( $6.2 \times 10^{10}$  TP/ml) which proves and supports the conclusion previously taken.

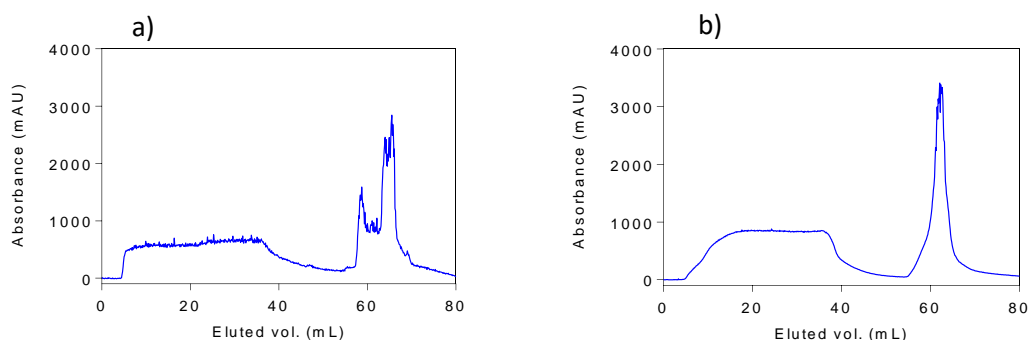


Figure 30 - Purification Validation Assays (wavelength 280 nm): 0 mM (a) and 200 mM (b)

#### 4.1.4 Polishing scouting

After AEX chromatography purification, a step of polishing is carried to further deplete impurities present. As this is a step closer to the final product, the quality control narrows and the outcome must fulfil the intended specifications.

A SEC resin performance evaluation consisted in a 500  $\mu$ l injection of AEX purified oncolytic virus in a pre-equilibrated column followed by fractionation and peak monitoring using UV detector at 280 nm (Fig. 31). The results were then analysed by NTA using NanoSIGHT and the impurity removal ratios by the standard analytical methods. Results indicate that a 56% virus recuperation was obtained.

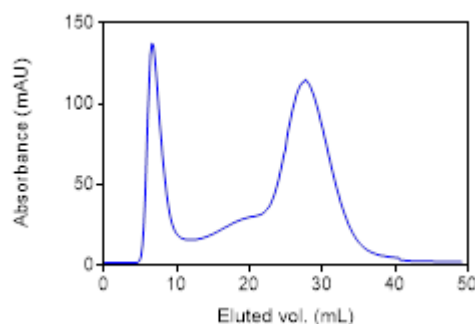


Figure 31 - Polishing Assay with a 500  $\mu$ l injection

Optimal loading amount was tested to maximize productivity without compromising quality. The loaded volumes and correspondent column volume are depicted in Figure 32 and summarized in Table 6. These runs were conducted at the recommended flow rate (4 ml min<sup>-1</sup>) and analysed with the same QC methods used before.

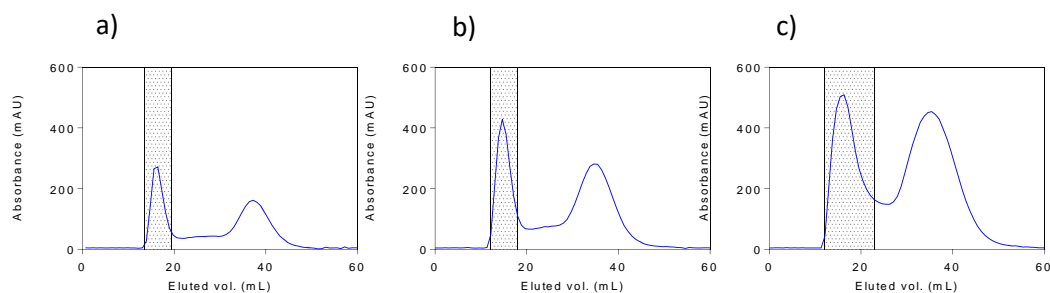


Figure 32 - Loading assays: 5% (a), 10% (b), 20% (c)

Table 6 - Loading assay

<b><u>Vsample</u></b> <b><u>Vcolumn</u></b>	Virus Recovery (%)	DNA removal (%)	Total protein removal (%)
<b>0.05</b>	69	96	67
<b>0.1</b>	65	94	80
<b>0.2</b>	70	97	72

According to these results and as suggested by previous research, the loading should be up to 20% of column volume. The virus recovery is stable between these values and the impurity removals average more than 95% of DNA removal and 70% of total protein.

After this analysis, several replications were conducted to ensure that the data obtained was correct. As an example, a validation run was performed following different process steps with comparable results. This involved a 5 ml injection, which corresponds to 14% of column's volume, and achieved similar impurity removal ratios (Fig. 33). The main difference experienced was the virus recovery yield that went to 90%. This factor can be explained by several aspects along the purification train because although the injected oncolytic viruses were the same, their

upstream or downstream routes could differ concerning bioreactor providence or elution conditions, respectively. With this being said, the results demonstrate this approach affectability and applicability for this purification process.

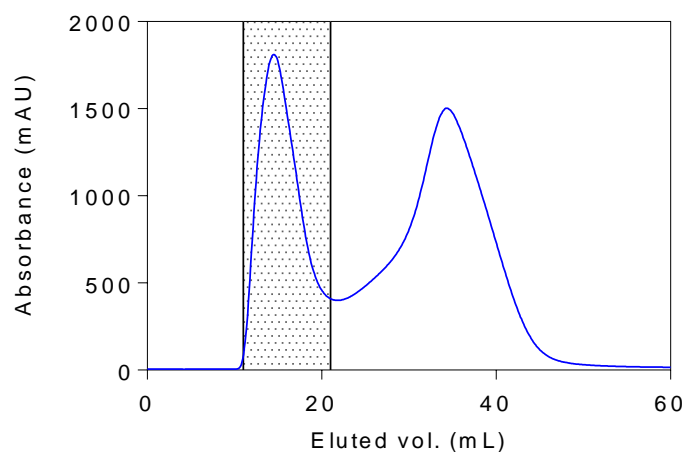


Figure 33 - Polishing Validation Run

#### 4.1.5 Sterile filtration

This last processing step consists of an impurity filtration step for final formulation in a sterile environment. The experiments were made using two different sterile filters, the first was a Millex GP Fibre Unit with a PES membrane and a 0.22  $\mu\text{m}$  pore size (Millipore Express, USA), the second was an Acrodisc PF with 0.8/0.2  $\mu\text{m}$  Supor Membrane (PALL, USA). The filtered materials were once again evaluated by HPLC-SEC method and impurity removal methods (Table 7). According to the results, a better virus recovery is obtained with Millipore's filter while a greater impurity removal is achieved by PALL's. At this stage, the results of other steps would greatly determine which parameter is more relevant to achieve quality parameters. In this scenario, virus recovery would be once again the factor of choice.

Table 7 - Sterile filtration assay

Membrane Filter	Virus Recovery (%)	DNA removal (%)	Total protein removal (%)
Millex GP	89	16	39
Acrodisc PF	81	17	58

## 4.2 Development of a multicolumn chromatography purification step

The single-column chromatographic experiments performed (see sub-chapter 4.1.4) presents initial results and parameter setpoints that can be used for further development. These conclusions can, at the same time, be employed in scale up procedures for pilot plan processes or in other scouting research methods like multi-column chromatography at the same scale. As mentioned before, the optimal loading quantity, elution condition and flow velocity have been addressed and will be considered for the following experimentations. The system used for this process is a two-column array using 0.967 ml columns connected a modified ÄKTA 10 system (Fig. 34).



Figure 34 - Two-column chromatography array

This setup allows for the main steps needed for column chromatography to be occurring in different columns at the same time. The display of operations overtime demonstrates the possibilities given by such procedure and the single column scheme evidences each flow inlet and recirculation path for this approach (Fig. 35 a, b).

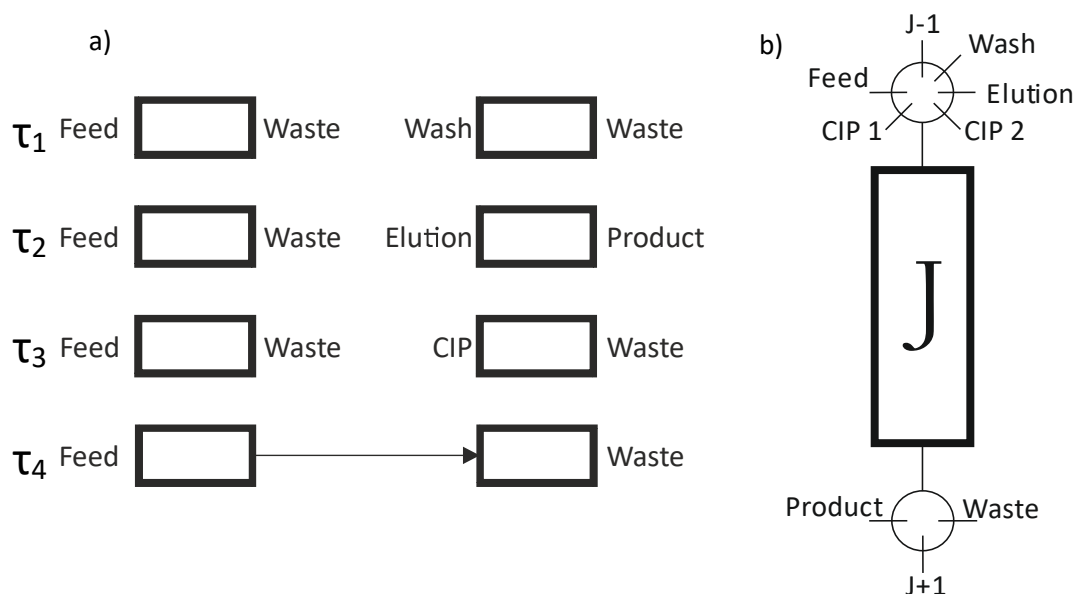


Figure 35 - Multicolumn scheme (a) and single column inlets (b)

The multi-column system presented shows that while the first column is being loaded the other is being prepared for it. This preparation includes wash, elution and clean-in-place procedures until both columns are connected. This scheme represents only half-cycle, being the next one symmetrical to the presented, with the inlet and outlet ports exchanging positions.

The single-column representation demonstrates the connections to previous and following columns or inlets/outlets within a generic multi-column arrangement, complemented with the discrimination of the main flows used. For the runs performed, the washing procedure consisted of the standard working buffer (50 mM HEPES filtrated solution with pH 7.5), the elution on a similar HEPES solution with 2 M of Sodium chloride added, and the CIP solutions were water and 1 M NaOH.

Before starting the oncolytic virus purification, a BSA and Tryptophan model solution was repeatedly loaded to evaluate system design and the control method implemented. These assays are relevant to make sure that all chromatography steps and timeframes are correctly assembled before starting the purification run.

This scouting experiment started with a breakthrough curve of the considered system using only one column to evaluated the correct loading amount (Fig. 36). After this, several experiences were made to optimize time frames and inlet flow rates.

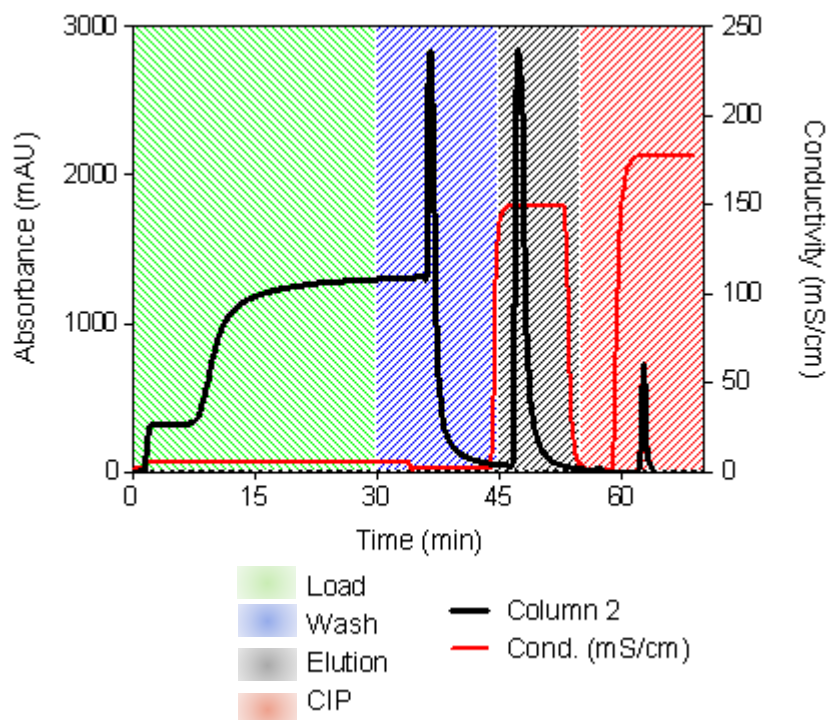


Figure 36- BSA and Tryptophan breakthrough

The step parameters used for each assay are depicted according to the flowrate used for each (Tables 8, 9, 10). The first and second runs allowed to mark limits for best loading quantity and establish the best balance between all flows at 3<sup>rd</sup> assay. Despite these adjustments, each run performed in a similar way and returned a periodic behaviour of the concentration profiles. After adjusting the baseline for each first step, the chromatograms of the last two half cycles demonstrate the overlay of runs which validates the accuracy of the system (Figures 37, 38).

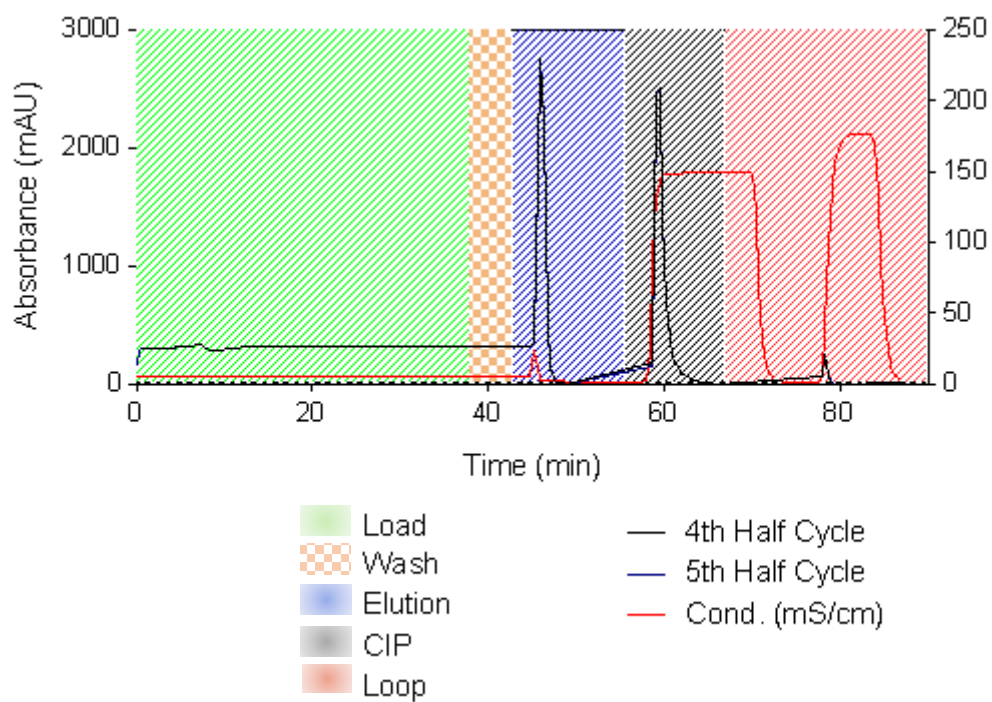


Figure 37 - BSA and Tryptophan Multicolumn Chromatography Assay 1

Table 8 - BSA and Tryptophan Multicolumn Chromatography Assay 1

Step	Duration (min)	$Q_{\text{Feed}}$ (ml/min)	$Q_{\text{Wash}}$ (ml/min)	$Q_{\text{Elution}}$ (ml/min)	$Q_{\text{CIP}}$ (ml/min)
1	5	0.4	2	0	0
2	5	0.4	0	2	0
3	7.5	0.4	0	0	2
4	10	0.4	0	0	0



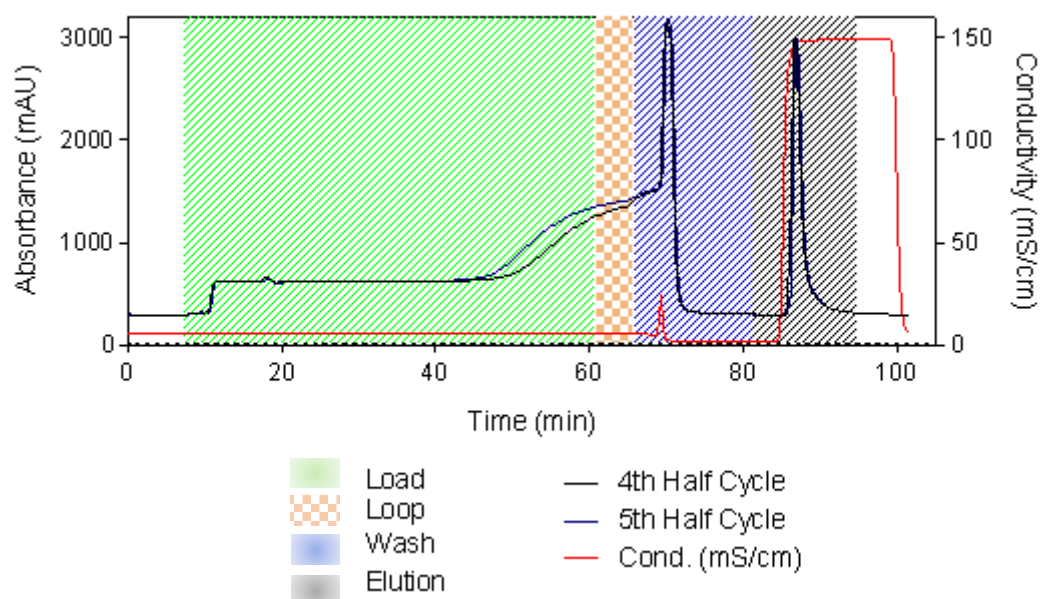


Figure 38 - BSA and Tryptophan Multicolumn Chromatography Assay 2

Table 9 - BSA and Tryptophan Multicolumn Chromatography Assay 2

Step	Duration (min)	$Q_{\text{Feed}}$ (ml/min)	$Q_{\text{Wash}}$ (ml/min)	$Q_{\text{Elution}}$ (ml/min)	$Q_{\text{CIP}}$ (ml/min)
1	5	0.67	2.33	0	0
2	5	0.67	0	2.33	0
3	7.5	0.67	0	0	2.33
4	10	0.67	0	0	0

With these results, it's possible to conclude that, despite applying different flow rates, the overlay of half-cycles suggests accuracy on the constructed method. Furthermore, these chromatograms make possible to evaluate the chosen time frames and adjust them to improve the process steps. The first assay, in which the sample loading was performed at 0.4 ml/min, didn't achieve a virus breakthrough and the flow was considered insufficient for the experiment. In the other end, the second experiment demonstrates a fully achieved breakthrough before washing starts. This situation reveals that the flow rate applied of 0.67 ml/min was too much for the system and should be brought down to prevent virus loss.

With this being said, the third experiment was adjusted to an intermediate loading flowrate to diminish the virus quantity in flow through and ensure the maximum amount of loading (Fig. 43). The loop section demonstrates the period from when the loaded j column is connected to the j+1, when it ends the first column is washed and the second is directly fed.

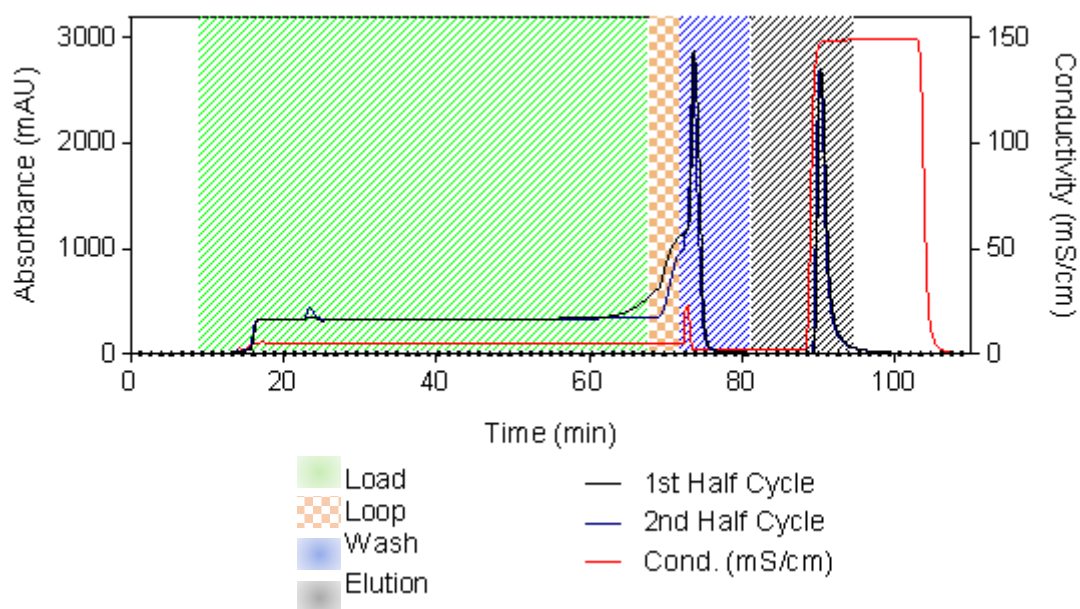


Figure 39 - BSA and Tryptophan Multicolumn Chromatography Assay 3

Table 10- BSA and Tryptophan Multicolumn Chromatography Assay 3

Step	Duration (min)	$Q_{\text{Feed}}$ (ml/min)	$Q_{\text{Wash}}$ (ml/min)	$Q_{\text{Elution}}$ (ml/min)	$Q_{\text{CIP}}$ (ml/min)
1	5	0.5	2.5	0	0
2	5	0.5	0	2.5	0
3	7.5	0.5	0	0	2.5
4	10	0.5	0	0	0

The last BSA and Tryptophan model achieved the intended objective and demonstrated that the method can operate consistently at the correct and pre-defined conditions. After this experimentation, the evaluated data could be introduced into a modelling software to implement a method that would theoretically describe the results obtained. This process would

require greater knowledge of the system and could imply further experimentation associated with time consuming procedures and analysis, and so it wasn't performed.

The following step was the introduction of adenovirus on the system (Fig. 40). The previous protein capture columns were replaced by AEX resin B columns and the method was also tested for different flowrates. This experiment started after total system sanitation of model assays residues with multiple volumes of water and sodium hydroxide before complete equilibration.

The approach taken followed the same steps as before; an initial breakthrough curve was accomplished with the monitored column to evaluate system periods and step time frames needed for correct completion. The results demonstrate a fully established BT curve and correct time frames for this analysis.

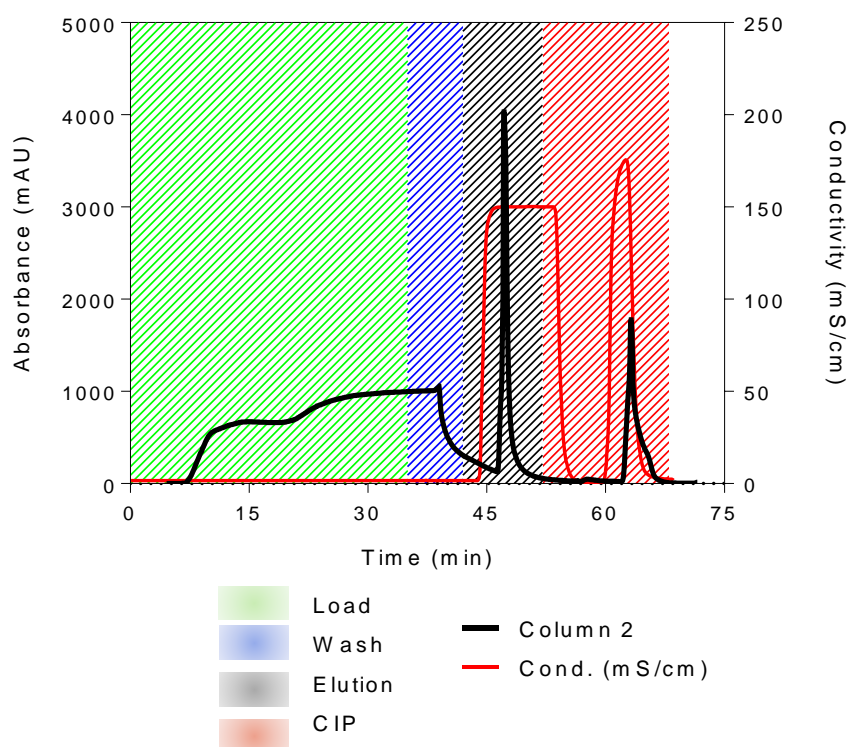


Figure 40 - Adenovirus breakthrough in AEX media B

With these outcomes, two process attempts were made to evaluate the best way to apply the oncolytic virus considering the previous batch experiments regarding DBC<sup>10%</sup> made with AEX media resin B. The first one was conducted at 0.67 millilitres per minute and resulted on an acceptable attempt with appropriate time frames for each step but still, a higher loading amount than recommended or wanted (Fig. 41). The second attempted achieve better results

due to flow reduction to 0.5 ml/min which diminished the amount of CV's loaded for the same time frame and the number of viruses wasted in flowthrough (Fig. 42).

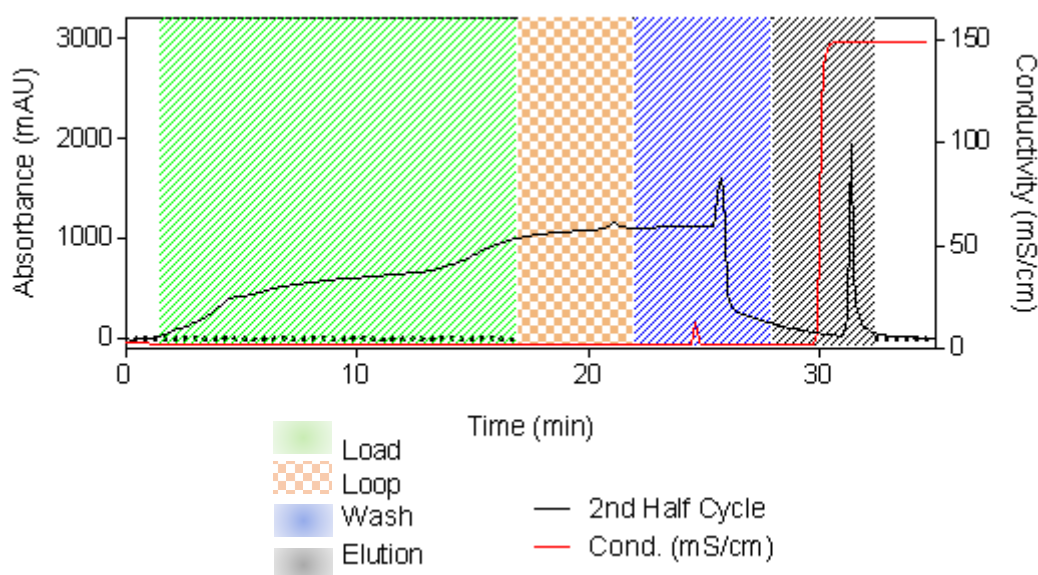


Figure 41 - Adenovirus multicolumn chromatography assay 1 (0.67 ml/min)

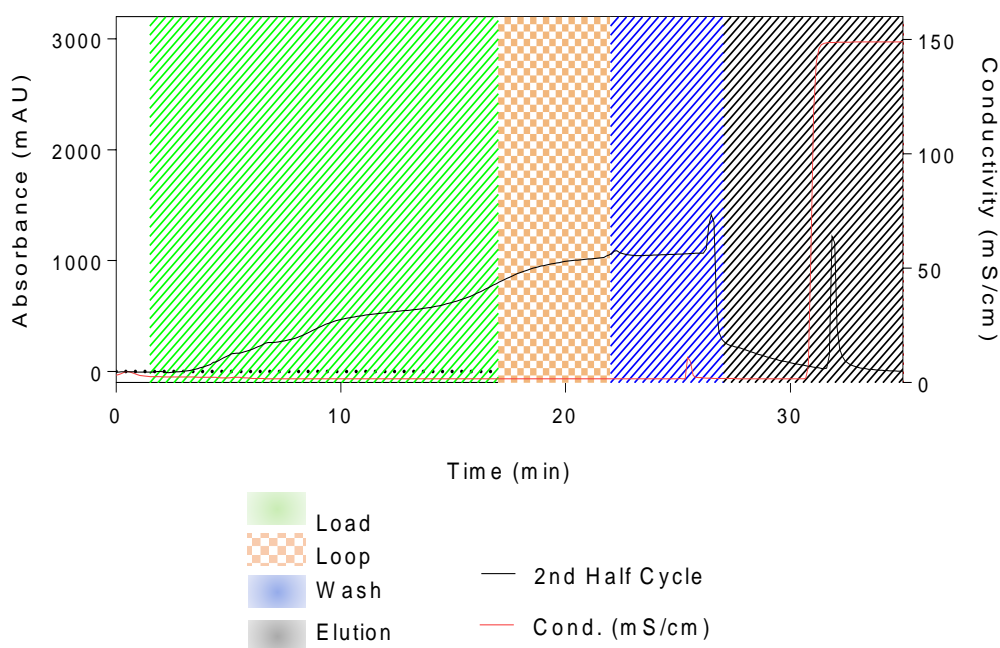


Figure 42 - Adenovirus multicolumn chromatography assay 2 (0.5 ml/min)

With these results, the completion of a multicolumn chromatography step for oncolytic purification is a possibility which may be worth integrating into the downstream train. This continuous procedure reduces buffer spending and significantly diminishes operation periods while ensuring maximum loading amount and reduction of virus flowthrough losses. The objective of this experimentations was accessing if this system could be applied to oncolytic

virus purification but further analysis is required to take conclusions and establish any relations between single-column and multi-column strategies.



# 5

# Conclusion and Future Work

*This chapter summarizes the accomplished results and establishes a bridge for further research with innovative approaches and different techniques.*





## 5.1 Conclusion

The extended research done throughout the downstream process displays the increasing interest in bioprocess investigation and the necessity for purification improvements that can significantly change the standard procedures. The presented work demonstrates several possible strategies for each step resolution and, even though some of them aren't the selected for this sequence, they certainly can be the best fitting for other approaches.

The evaluations made allowed for a supported arrange of stratagems that can deliver the purified oncolytic virus with the correct specification parameters required.

The initial experimentation focused on clarification and the establishment of a 2-step filtration which guarantees high virus recovery and relevant impurity removal that results from extraction in the bioreactor. The following ultra and diafiltration assays result on a scale dependent decision with the focus on an automated procedure that is reliable and accurate, but most importantly independent. These steps ended with a fully established line of operation with good outcomes for purification experiments.

The chromatography strategies differentiate between relevant ligand Q chromatography medias. After single column experimentations, the multicolumn approach was evaluated as an alternative for the standard route and shown great applicability for this purification train. The upcoming polishing was made through a SEC column with valuable outcome results concerning virus yield, but the multimodal resin tested could be a strong possibility for future endeavours if improvements to it are made. The sterile filtration that followed ensured a pure and controlled environment for the product and achieved the required quality parameters.

In parallel, the implementation of an analytical method for particle analysis increases the range of measurements possible as it allows accurate and linear results that can sustain an entire development strategy with a simple operation procedure.

With all things considered, this research led to a complete purification train with 53% yield for oncolytic viruses (Fig. 43) that fulfils excellence requirements but leaves a margin for further investigation, improvement and innovation.

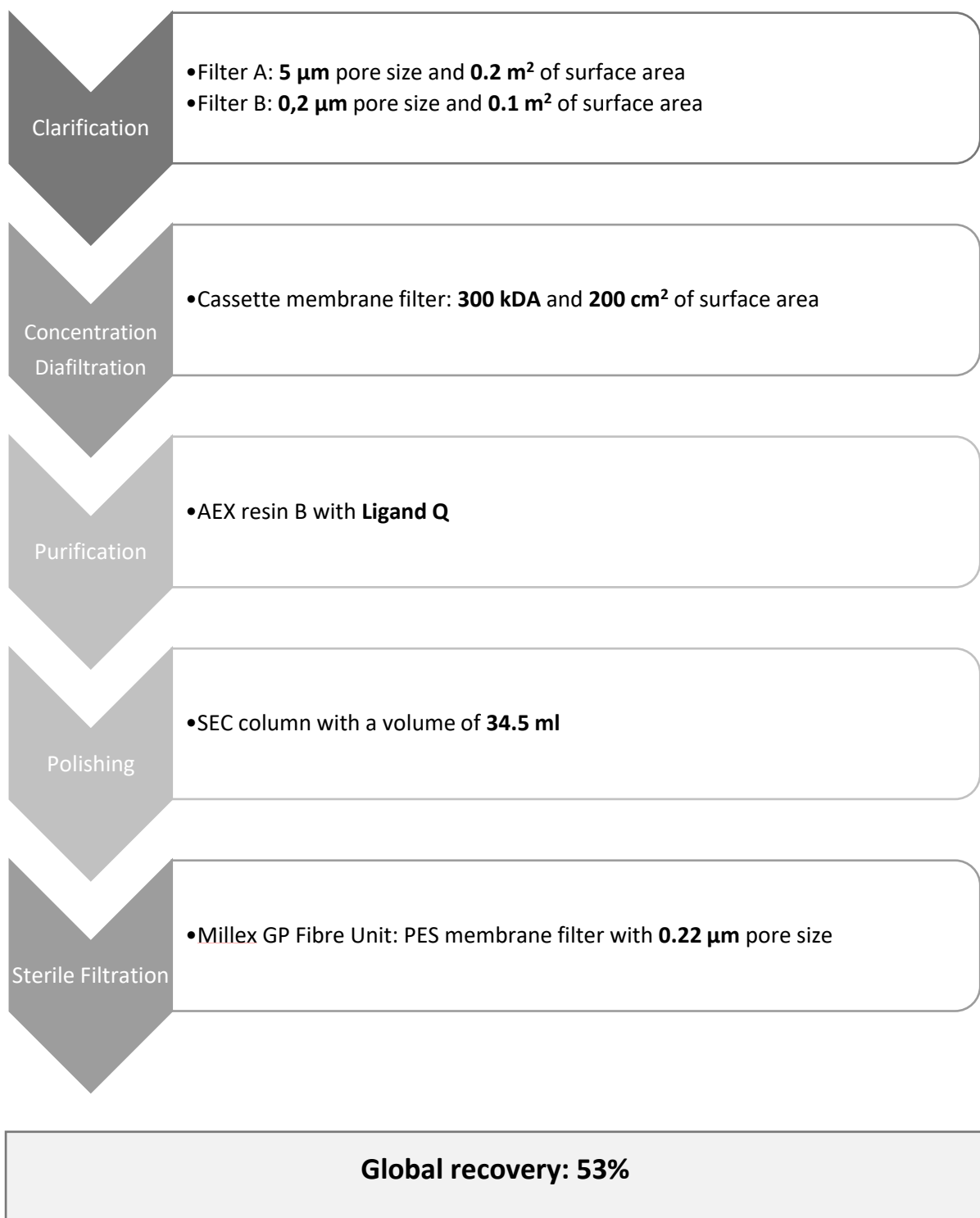


Figure 43 - Downstream purification diagram

## 5.2 Future Work

The work developed and reported in this thesis introduced new points of view to solve the existent problems throughout the downstream train. For a short term, the introduction of a fully automated ultrafiltration device is an accomplishable objective that could differentiate and facilitate the entire process. As a mean to achieve it, a full understanding of equipment's capabilities and limitations is needed in parallel with multiple biological system testing with several filtration membranes, volumes and conditions.

In the midterm, other innovative strategies could be implemented. As an example, the wave reactor used for cell growth, and then virus production, is a batch reactor and despite having its conditions assessed or continuously adjusted has its downfalls. Another approach could be the introduction of a perfusion reactor that allows gradual nutrient introduction and bulk monitoring associated with steady product release. With this mechanism, a more complex operation is needed, but considering it ensures maximum viability and productivity by countering excess concentrations and growth limitations, it might be a worthy alternative. This process highly increases cell density which requires innovative downstream strategies to deal with it and a complete makeover on the purification train.

Concerning multicolumn chromatography, it requires more investigation and additional development in order to become a stable alternative for oncolytic viruses. The direct improvement relies on a chromatographic equipment designed specifically for multicolumn that ensures a capable operation method and a wide range of possibilities.

Another significant development that could be considered relates to process control. The usual static system relies on predefined switch times between columns. By relying on time control, the arrangement may experience a downsize on its outcomes from not considering possible variability in feed concentration or different resin binding capacity. A better approach might rely on a control strategy that introduces the possibility of adjusting specific operation time frames by real time monitoring the outcome and comparing it with intended. Even though it would be comparatively more complex, this real-time adaptive control could ensure overall optimization and improvements.

The long run development would be the execution of a full continuous purification strategy associated to the perfusion reactor described above, and a series of different capability columns that could, in a single operation, deliver the sterile and purified adenovirus without step interruptions and product losses.



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